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REGULATION OF MITOGEN-ACTIVATED PROTEIN KINASE AND PHOSPHOINOSITIDE 3-KINASE SIGNALING BY WILD-TYPE AND ONCOGENIC RAS

by

Amy Young

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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of the

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by

Amy Young

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It is hard to believe that my graduate career has finally come to an end. The work presented here could not have been possible without the support and generosity of the many people I have had the privilege to work with during my time at UCSF.

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REGULATION OF MITOGEN-ACTIVATED PROTEIN KINASE AND PHOSPHOINOSITIDE 3-KINASE SIGNALING BY WILD-TYPE AND ONCOGENIC RAS

by

Amy Young

ABSTRACT

Ras is a small GTPase that functions as a binary molecular switch, cycling between an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state. It is estimated that 30% of human tumors carry an oncogenic somatic mutation in one of three Ras isoforms. Oncogenic *RAS* alleles differ from their wild-type counterpart by a single missense point mutation that results in an amino acid substitution at position 12, 13, or 61. These amino acid substitutions impair the ability of GTPase activating proteins (GAPs) to facilitate GTP hydrolysis and consequently result in deregulated Ras signaling. Oncogenic Ras constitutively activates downstream effector pathways, including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, and promotes the uncontrolled growth, proliferation, and survival of cancer cells. In cancers for which there is no *RAS* mutation, increased signaling through the pathway is achieved by amplification or activation of upstream receptor tyrosine kinases, loss-of-function mutation in negative regulators, or activating mutation of downstream effectors, illustrating the importance of high pathway activity in the pathogenesis of cancer.

The oncogenic Ras isoform is thought to be primarily responsible for mediating the activation of downstream signaling pathways, although the two wild-type Ras isoforms remain functional. In fact, the signaling contribution of wild-type Ras in this context has largely been discounted and

unexamined. The work presented here uncovers a novel role for wild-type Ras signaling in cancer cells with oncogenic *RAS* mutations. Wild-type Ras can be activated by external growth cues and inactivated by intrinsic regulatory proteins, and the modulation of wild-type Ras activity results in fluctuations in downstream signaling, despite the presence of oncogenic Ras. Additionally, this work demonstrates a critical role for wild-type Ras signaling in the proliferation of cancer cells expressing oncogenic *RAS*. These studies expand our understanding of the intricacies of Ras signaling and may uncover less obvious modes of targeting the pathway that have yet to be exploited.

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CHAPTER 1.

INTRODUCTION

1.1. THE RAS FAMILY OF GTPASES

In the late 1970s and early 1980s, the *ras* genes were identified as the oncogenes responsible for the transforming properties of the Harvey (*HRAS*) and Kirsten (*KRAS*) rat sarcoma viruses (Chang et al., 1982; DeFeo et al., 1981; Ellis et al., 1982). In 1982, constitutively active mutant forms of H-Ras and K-Ras were identified in human bladder, lung and colon cancer cell lines (Der et al., 1982; Parada et al., 1982; Santos et al., 1982). Subsequent studies in neuroblastoma and leukemia cells led to the isolation of a third Ras isoform, termed *NRAS* (Hall et al., 1983; Shimizu et al., 1983; Taparowsky et al., 1983).

H-Ras, K-Ras, and N-Ras are the founding members of a superfamily of monomeric small GTPases that are characterized by the ability to bind and hydrolyze guanine nucleotides. The Ras superfamily is comprised of more than 150 proteins that can be subdivided into five subfamilies (Ras, Rho, Rab, Arf, Ran) based on similarity in both sequence and function (Vigil et al., 2010). In their active GTP-bound state, members of the Ras superfamily regulate diverse cellular functions, including gene expression, actin organization and vesicular trafficking. This work will focus specifically on the role of the three canonical Ras isoforms (H-Ras, K-Ras and N-Ras) in integrating extracellular growth cues to initiate intracellular signaling cascades.

1.1.i. Ras structure and function

Three cellular *RAS* genes encode four 21 kiloDalton (kDa) proteins: H-Ras, N-Ras, K-Ras 4A and K-Ras 4B. The *KRAS* gene is alternatively spliced at the C-terminus to generate both K-Ras 4A and the predominant splice isoform K-Ras 4B. The four proteins are highly homologous at the N-terminal G domain (amino acids 1-165), but diverge at the C-terminal hypervariable (HVR) domain (amino acids 165-188/189). The portion of the protein that specifies nucleotide binding (amino acids 1-85) is identical across all isoforms, and is comprised of the P loop (phosphate-binding loop; amino acids 10-16), switch I (amino acids 32-38) and switch II (amino acids 59-67) regions. The P loop mediates binding to the γ-phosphate of GTP, and the switch I and II regions

mediate binding to Ras regulators and effectors. The four isoforms share 85-90% sequence identity over the next 80 amino acids (amino acids 85-165), but diverge at the C-terminal HVR domain (amino acids 165-188/189). Post-translational modifications of key residues in the HVR domain are required to target Ras to the inner leaflet of the plasma membrane. Each isoform is farnesylated at the C-terminal CAAX motif, in which C is a cysteine, A is usually an aliphatic amino acid, and X represents any amino acid. The farnseylation is followed by proteolytic cleavage of the -AAX sequence. Although modification of the CAAX motif is required for proper Ras localization, an additional second signal is required for full membrane recruitment. The so-called second signal in H-Ras, N-Ras and K-Ras 4A involves palmitoylation at cysteine residues upstream of the CAAX motif. The hydrophobic palmitates complement the hydrophobic farnesyl moiety and aid in securing the Ras proteins in the plasma membrane. In contrast, the second signal in K-Ras 4B involves a stretch of polybasic lysine residues upstream of the CAAX motif. K-Ras 4B becomes firmly anchored in the plasma membrane when the electropositive lysine residues in this region form ionic bonds to the electronegative lipid head groups of the plasma membrane (Karnoub and Weinberg, 2008; Schubbert et al., 2007). Plasma membrane localization is required for proper biological activity of both wild-type and oncogenic Ras (Srivastava et al., 1985; Willumsen et al., 1984a; Willumsen et al., 1984b).

Genetic studies in mice have uncovered an important role for K-Ras 4B in development. K-Ras 4B deficient mice die of anemia, fetal liver erythropoiesis and cardiac defects at days 12-14 of gestation (Johnson et al., 1997; Koera et al., 1997). In contrast, K-Ras 4A deficient mice develop normally, as do mice that are deficient in H-Ras and/or N-Ras (Esteban et al., 2001; Umanoff et al., 1995). These data suggest that K-Ras 4A, H-Ras and N-Ras may have redundant or overlapping roles in development. In a model in which the coding sequence for K-Ras is replaced with that of H-Ras, mice are born at the expected Mendelian frequencies, suggesting that H-Ras can compensate for K-Ras in embryonic development when controlled by the K-Ras promoter (Potenza et al., 2005). Although H-Ras can functionally compensate for K-Ras during embryonic development if expressed in the proper tissue compartments, a high percentage of adult mice

ultimately develop cardiomyopathies, suggesting H-Ras cannot functionally replace K-Ras during adult life.

A systematic investigation of *Ras* expression in the mouse revealed that *Hras* is predominantly expressed in the brain, muscle and skin. In contrast, *Kras* is highly expressed in the lung, gut and thymus. Finally, *Nras* expression is highest in the thymus and testes (Leon et al., 1987). Despite variations in the levels of each transcript, *RAS* genes are concurrently and ubiquitously expressed in most mouse and human tissues.

1.1.ii. Activation of Ras signaling

Ras functions as a binary molecular switch, cycling between an inactive GDP-bound state and an active GTP-bound state. In the basal state, Ras is predominantly bound to GDP. Mitogenic growth factors activate receptor tyrosine kinases located at the cell surface, resulting in the recruitment of signaling and adaptor proteins to the intracellular portion of the activated receptor. Guanine nucleotide exchange factors (GEFs) are among the proteins recruited to the plasma membrane by activated receptors. GEFs promote the exchange of bound GDP for GTP on nearby Ras molecules, thereby activating the Ras signaling cascade (Figure 1.1).

The mechanism by which GEFs promote the exchange of bound GDP for GTP was gleaned from the structural characterization of Ras bound to a well known GEF called Son of sevenless homolog 1 (SOS1) (Boriack-Sjodin et al., 1998). SOS1 inserts an α-helix that causes displacement of switches I and II of Ras, thereby disrupting nucleotide binding and causing GDP dissociation. Because cellular levels of GTP are estimated to be 10-fold higher than GDP, GTP predominantly re-binds the nucleotide binding pocket of Ras. SOS1 also contains an allosteric Ras-GTP binding site that stabilizes the binding of Ras-GDP to the catalytic site of SOS1, thereby creating a positive feedback loop to amplify Ras activation (Margarit et al., 2003). GTP binding induces a conformational change in Ras that increases its affinity for effector proteins. The

interaction of GTP-bound Ras with effector proteins initiates signaling cascades to promote cellular growth, proliferation, and survival.

1.1.iii. Regulation of Ras signaling

The intrinsic rate at which Ras hydrolyzes the terminal phosphate of GTP is slow, and is estimated to occur about once per hour (Schubbert et al., 2007). GTPase activating proteins (GAPs) increase the rate at which Ras hydrolyzes GTP to GDP by several orders of magnitude. The hydrolysis of bound GTP to GDP returns Ras to its basal, inactive conformation and terminates downstream signaling (Figure 1.1).

The mechanism by which GAPs promote the hydrolysis of bound GTP to GDP was gleaned from the structural characterization of Ras bound to the catalytic domain of a well known GAP called p120 RasGAP (Scheffzek et al., 1997). The arginine 789 residue of p120 RasGAP (termed the "arginine finger") inserts into the P-loop of Ras and stabilizes the transition state of the hydrolysis reaction by neutralizing the negative charge at the γ-phosphate, thereby stimulating GTP hydrolysis. In addition, the glutamine 61 residue of Ras coordinates the attacking water molecule, and forms a hydrogen bond with the arginine finger of p120 RasGAP. Importantly, the arginine finger is conserved in other GAPs as well (Bos et al., 2007; Schubbert et al., 2007).

Eight mammalian GAPs capable of stimulating Ras GTPase activity have currently been described: p120 RasGAP, neurofibromin, GAP1^{1P4BP}, Ca^{2+} -promoted Ras inactivator (CAPRI), Ras GTPase activating-like protein (RASAL), DAB2IP, SynGAP and nGAP (Grewal et al., 2011). The GAP-related domain (GRD) responsible for catalytic activity is conserved across all of these proteins (Bos et al., 2007).

1.1.iv. Aberrant Ras signaling in cancer

It is estimated that 30% of human tumors carry an oncogenic somatic mutation in one of the three Ras isoforms (Forbes et al., 2011). Oncogenic *RAS* alleles differ from their wild-type counterpart by a single missense point mutation that results in an amino acid substitution at position 12, 13, or 61 (Schubbert et al., 2007). These amino acid substitutions impair the rate of GAP-mediated GTP hydrolysis and consequently result in hyperactive Ras signaling. Mutation at position 61 of Ras abolishes the GAP-mediated hydrolysis of GTP by destabilizing the interaction between Ras and the arginine finger of GAPs (Der et al., 1986). Substitution of glycine 12 or 13 with any other amino acid other than proline also impairs GAP-mediated GTP hydrolysis. The bulky side chain of the substituted amino acid at position 12 sterically blocks the proper orientation of glutamine 61 with the arginine finger of GAPs and subsequently destabilizes the transition state of the GTP hydrolysis reaction (Colby et al., 1986; Franken et al., 1993).

The incidence of mutation at these hotspots varies among the three Ras isoforms. Mutations at position 12 or 13 of *KRAS* account for nearly 99% of those detected. In contrast, the majority of *NRAS* mutations are located at position 61 (60%), though a significant proportion of mutations are also detected at positions 12 and 13 (24.4% and 12.7%, respectively). In the case of *HRAS*, the pattern of mutation is more widely distributed, with 54% of mutations occurring at position 12, 34.5% at position 61 and 9% at position 13 (Castellano and Santos, 2011; Fernandez-Medarde and Santos, 2011; Forbes et al., 2011).

KRAS is by far the most frequently mutated Ras isoform, and is often mutated in cancers of the colon, pancreas and lung. In contrast, *NRAS* mutation is often observed in hematological malignancies and in melanoma. *HRAS* is the most frequently mutated Ras isoform in bladder cancer, although its overall rate of mutation in cancer is quite low in comparison to the other two Ras isoforms (Castellano and Santos, 2011; Fernandez-Medarde and Santos, 2011; Forbes et al., 2011; Schubbert et al., 2007).

In cancers for which there is no *RAS* mutation, increased signaling through the pathway is achieved by amplification or activation of upstream receptor tyrosine kinases, loss-of-function mutation in negative regulators, or activating mutation of downstream effectors, illustrating the importance of high pathway activity in the pathogenesis of cancer (Downward, 2003)

1.1.v. Therapeutic targeting of Ras signaling in cancer

Developing therapeutic agents to directly block oncogenic Ras activity has thus far been a challenging and unsuccessful endeavor. Efforts to directly target oncogenic Ras with nucleotide analogs have been discouraged due to the high cellular GTP concentration and the picomolar affinity of Ras for GTP. Efforts to prevent the recruitment of Ras to the plasma membrane by targeting post-translational processing enzymes have also been unsuccessful, due to the fact that both N-Ras and K-Ras can be targeted to the plasma membrane through alternate mechanisms. Another therapeutic approach involves silencing the expression of oncogenic Ras by RNA interference (RNAi). As with other RNAi-based therapies, the promise of this approach depends on efficient small interfering RNA (siRNA) delivery, uptake, and gene silencing (Gysin et al., 2011).

Consequently, a great deal of effort has been put forth to develop therapies targeting effector pathways downstream of Ras. Constitutive activation of downstream effector pathways by oncogenic Ras results in the uncontrolled growth, proliferation, and survival of cancer cells. Understanding which effector pathways are required for Ras-driven oncogenesis is critical for determining which pathways should be targeted for therapeutic purposes. Many Ras effector pathways are comprised of kinase cascades, providing multiple nodes for potential therapeutic intervention. While several Ras effectors have been identified and comprehensively described (Downward, 2003; Karnoub and Weinberg, 2008; Repasky et al., 2004), two of the best characterized Ras effector pathways are the MAPK and PI3K signaling pathways. Importantly, both pathways are integral to Ras-driven transformation, and small molecule compounds

targeting these pathways are currently under clinical investigation (Courtney et al., 2010; Pratilas and Solit, 2010).

1.2. THE MAPK PATHWAY

The mitogen-activated protein kinase (MAPK) signaling cascade was the first Ras effector pathway identified. Raf serine/threonine kinases (A-Raf, B-Raf, and C-Raf/Raf-1) specifically interact with GTP-bound Ras, resulting in the activation of Raf protein kinase activity (Karnoub and Weinberg, 2008; Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). Upon activation by Ras, Raf phosphorylates and activates the serine/threonine kinase MEK, which in turn phosphorylates and activates the serine/threonine kinase ERK. This series of signaling events results in the activation of transcriptional regulators that promote a wide variety of cellular events, including cell cycle progression and cell proliferation (Downward, 2003; Karnoub and Weinberg, 2008; Schubbert et al., 2007).

Raf activation is a complex multi-step process that is still not completely understood. Inactive Raf isoforms are located within the cytosol in an autoinhibited state. The N-terminal regulatory domain of Raf binds to the C-terminal kinase domain in an autoinhibitory fashion. 14-3-3 dimers stabilize the autoinhibited conformation by binding phosphorylation sites within the N- and C-terminal domains. Active Ras-GTP interacts with two sites within the N-terminal conserved region 1 (CR1) of Raf: Ras binds the Ras-binding domain (RBD) of Raf in a GTP-dependent manner, and also forms secondary interactions with the cysteine rich domain (CRD) of Raf in a nucleotide-independent manner. Ras activation mediates the recruitment of Raf to the plasma membrane, which induces the release of 14-3-3 from the N-terminal regulatory region and facilitates the phosphorylation and activation of the C-terminal Raf kinase domain (Ritt et al., 2010; Wellbrock et al., 2004).

The requirement for MAPK signaling in Ras-mediated transformation and tumorigenesis has been well established (Cuadrado et al., 1993; Karnoub and Weinberg, 2008; Khosravi-Far et al., 1995; Khosravi-Far et al., 1996; White et al., 1995). Dominant negative mutants of Raf-1, MEK and ERK inhibit Ras-driven transformation, highlighting the importance of this signaling cascade downstream of Ras (Cowley et al., 1994; Kolch et al., 1991; Schaap et al., 1993; Westwick et al., 1994). In support of these findings, mutations in the effector loop of H-Ras V12 that abrogate its ability to bind Raf-1 eliminate its transforming potential in mammalian cells, demonstrating the requirement for Raf-1 activity downstream of activated Ras (White et al., 1995). In addition, the growth inhibition induced by overexpression of dominant negative Ras N17 can be overcome by expression of constitutively active Raf-1 (Feig and Cooper, 1988). Finally, cells that lack Ras proteins altogether can be rescued from growth arrest by expression of activated forms of Raf, MEK or ERK proteins, again demonstrating that the MAPK pathway lies downstream of Ras (Drosten et al., 2010).

Baccarini and colleagues recently demonstrated that Raf-1 is required for the initiation and maintenance of squamous cell carcinoma in two separate models of Ras-driven tumorigenesis (Ehrenreiter et al., 2009). In the first model, Ras activation is achieved through a classical chemical carcinogenesis protocol, in which tumors are initiated through the topical application of 7,12-dimethylbenz[a]anthracene (DMBA), which causes an activating mutation in codon 61 of H-Ras. Tumor development is then promoted through the topical application of 12-O-tetradecanoylphorbol 13-acetate (TPA). In the second model, activation of the Ras pathway is achieved by expression of a dominant active form of SOS1 specifically in the epidermis. In both models, ablation of Raf-1 leads to the regression of established Ras-driven tumors, suggesting that Raf-1 might serve as an appropriate target for therapeutic intervention downstream of activated Ras. Interestingly, in these models the ability of Raf-1 to promote and maintain skin tumors is dependent on the inhibition of the RhoGTPase target Rok-α rather than the activation of the canonical MEK/ERK signaling cascade.

Recent studies demonstrate the importance of Raf-1 in the initiation of non-small cell lung cancer (NSCLC) driven by K-Ras^{G12D}. Barbacid and colleagues tested the requirement of different MAPK pathway components in a mouse model of NSCLC driven by oncogenic K-Ras^{G12D}. Combined ablation of Erk1 and Erk2 blocked tumor development, as did combined ablation of Mek1 and Mek2. While B-Raf was dispensable for tumor development, Raf-1 was essential for the initiation of K-Ras^{G12D} –driven NSCLC (Blasco et al., 2011). Tuveson and colleagues also found that ablation of Raf-1, but not B-Raf, impedes the formation of K-Ras^{G12D} –driven lung tumors (Karreth et al., 2011). These data suggest that B-Raf cannot compensate for Raf-1 in this model of lung tumorigenesis, and suggest that signaling from oncogenic K-Ras to MEK is primarily mediated by the Raf-1 isoform. The studies also suggest that Raf-1 is a suitable therapeutic target in tumors driven by oncogenic K-Ras, and warrant the development of Raf-1-selective kinase inhibitors.

Activating mutations in various components of the MAPK signaling cascade have recently been identified in patients with related genetic developmental disorders (Schubbert et al., 2007). Germline gain-of-function mutations in *KRAS*, *BRAF*, *MEK1*, and *MEK2* have been observed in patients with Cardiofaciocutaneous (CFC) syndrome (Niihori et al., 2006; Rodriguez-Viciana et al., 2006). Additionally, activating mutations in MAPK pathway components are present in patients with similar neuro-cardio-facial-cutaneous syndromes, including Noonan, LEOPARD, and Costello syndromes (Aoki et al., 2008; Denayer et al., 2008). In contrast, loss-of-function mutations in two negative regulators of the MAPK pathway, neurofibromin and Spred-1, are hallmarks of the developmental disorders NF1 and Legius Syndrome, respectively (Ballester et al., 1990; Brems et al., 2007; Cawthon et al., 1990; Martin et al., 1990). Taken together, these findings provide genetic evidence that the MAPK pathway functions downstream of Ras.

1.2.i. MAPK pathway mutations in cancer

Activating mutations in *BRAF* are commonly found in melanoma, colorectal, ovarian, and papillary thyroid carcinomas. Over 30 cancer-associated somatic missense mutations in *BRAF* have been identified. These mutations are often located within the glycine-rich nucleotide binding loop (P loop) or the N-terminal portion of the activation segment (Hubbard, 2004; Wan et al., 2004; Wellbrock et al., 2004).

Most *BRAF* mutations disrupt the autoinhibited state of the kinase and subsequently result in increased kinase activity. The V600E mutation is by far the most common cancer-associated *BRAF* mutation. The valine residue located at position 600 of B-Raf is located within the activation segment and makes contacts with a phenylalanine residue at position 467 in the P loop, thereby stabilizing the inactive state of the kinase. A substitution of glutamic acid for valine at this position promotes catalytic activity by destabilizing the inactive state and allowing the protein to adopt a conformation more reminiscent of the active state (Hubbard, 2004; Wan et al., 2004).

Surprisingly, a subset of *BRAF* mutations results in impaired kinase activity. Impaired kinase mutants often harbor amino acid substitutions that impair adenosine triphosphate (ATP) binding. It is hypothesized that these mutants are able to increase signaling through the MAPK pathway by transactivating Raf-1 kinase activity (Hubbard, 2004; Wan et al., 2004).

The identification of activating *BRAF* mutations in cancer further supports a role for MAPK signaling in oncogenesis (Davies et al., 2002; Dhomen and Marais, 2007). Interestingly, in melanoma and colorectal cancer a pattern of mutual exclusivity between *RAS* and *BRAF* mutation has emerged, suggesting that mutation of either gene may be functionally equivalent in the pathogenesis of these malignancies (Rajagopalan et al., 2002). However, in the case of *BRAF* mutation, activation of additional oncogenic signaling pathways such as the PI3K pathway may also be required (Tsao et al., 2004).

1.2.ii. Therapeutic targeting of the MAPK pathway in cancer

Attempts to target the MAPK signaling pathway for therapeutic purposes have focused largely on the development of Raf and MEK kinase inhibitors. Sorafenib (Nexavar) was the first Raf kinase inhibitor to be tested in clinical trials and is now FDA approved for the treatment of renal cell carcinoma and hepatocellular carcinoma (Wilhelm et al., 2006). Though Sorafenib was designed to inhibit Raf-1 kinase activity, it also has activity against additional cancer targets including VEGF-R2, PDGFR, Flt-3, c-kit, and FGFR-1 (Wilhelm et al., 2004). In fact, the success of Sorafenib as a cancer therapy has largely been attributed to its inhibitory effects on tumor angiogenesis, particularly for renal cell carcinoma, which is largely driven by hyperactive VEGF-R signaling (Wilhelm et al., 2006). In support of this, the VEGF-R2 inhibitor Sutent is equally effective in treating this disease. In contrast, Sutent failed to show efficacy in hepatocellular carcinoma, suggesting that Sorafenib's effects in this disease may indeed be mediated through inhibition of Raf kinase. Furthermore, clinical responses to Sorafenib correlate well with levels of MAPK signaling in this disease (Abou-Alfa et al., 2006). Activation of this pathway in hepatocellular carcinoma is caused by loss of negative regulatory proteins of the Spred and Sprouty families rather than by oncogenic Ras (Fong et al., 2006; Yoshida et al., 2006).

When B-Raf was identified as a major oncogene in human cancers, Sorafenib was tested for clinical efficacy in this disease (Davies et al., 2002). However, no clinical benefit was observed. This may be because Sorafenib interacts with the inactive form of Raf kinase, and is less effective against B-Raf V600E than wild-type B-Raf. This prompted the development of second generation Raf inhibitors, which demonstrate elevated specificity for B-Raf V600E (Li et al., 2010; Pratilas and Solit, 2010). While these inhibitors potently suppress MAPK signaling and cell growth in cancer cells expressing B-Raf V600E, they paradoxically have the opposite effect in cancer cells with wild-type B-Raf, including those with oncogenic *RAS* mutations (Cichowski and Janne, 2010; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). The promotion of MAPK signaling in *RAS* mutant cancer cells by Raf inhibitors has been reviewed extensively and

precludes the use of these inhibitors for the treatment of *RAS* mutant cancers (Cichowski and Janne, 2010; Cox and Der, 2010; Pratilas and Solit, 2010). Furthermore, though the use of the pan-RAF inhibitor PLX-4032 in melanoma patients harboring *BRAF* V600E mutations produced promising clinical results (Bollag et al., 2010; Flaherty et al., 2010; Lee et al., 2010), recent studies have identified multiple mechanisms of Raf inhibitor resistance, including enhanced receptor tyrosine kinase signaling as well as mutational activation of *NRAS* (Johannessen et al., 2010; Nazarian et al., 2010; Villanueva et al., 2010). The finding that mutational activation of *NRAS* can bypass the effects of Raf inhibition suggests that targeting Raf in the context of an activating Ras mutation may not be beneficial, despite the evidence in cell culture and animal models that suggest otherwise.

Several small molecule compounds have been developed to potently and selectively inhibit the activity of MEK. Studies in cancer cell lines and animal models demonstrate that *BRAF* mutation predicts sensitivity to these agents, although a subset of *RAS* mutant cell lines display sensitivity as well (Solit et al., 2006; Sos et al., 2009). Despite these promising preclinical results, the outcome of early clinical trials was underwhelming, in part due to the limited bioavailability and dose-limiting toxicity of the compounds (Fremin and Meloche, 2010; Pratilas and Solit, 2010). Several compounds with improved pharmaceutical properties are currently under clinical investigation, and hold promise for the treatment of *RAS* mutant tumors (Fremin and Meloche, 2010). Defining the factors that underlie MEK inhibitor sensitivity and resistance in *RAS* mutant cancers is of great interest, and will aid in determining which patients will benefit most from therapy.

1.3. THE PI3K PATHWAY

The phosphoinositide 3-kinase (PI3K) pathway is another well-studied signaling cascade downstream of Ras. Class IA PI3Ks are heterodimeric lipid kinases comprised of a p85 regulatory subunit and a p110 catalytic subunit. The p110 catalytic subunit of PI3K was identified

as a Ras effector when it was found to preferentially associate with GTP-bound Ras through its Ras-binding domain (RBD) (Pacold et al., 2000; Rodriguez-Viciana et al., 1994). Though PI3K can be activated by upstream receptor tyrosine kinases in a Ras-independent manner, association with and activation by Ras-GTP has proven to be a principal mechanism of PI3K regulation. PI3K catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). A primary downstream effector of PIP_3 is the serine/threonine kinase Akt, which activates a host of signaling programs to promote cell growth, survival, and migration (Wong et al., 2010; Yuan and Cantley, 2008).

1.3.i. PI3K pathway mutations in cancer

PI3K signaling is often up-regulated in tumor cells, indicating its importance in the pathology of cancer. Hyperactivation of the pathway can be achieved through a variety of mechanisms, including gain-of-function mutation in *PIK3CA*, which encodes the p110α catalytic subunit of PI3K (Bellacosa et al., 1995; Cheng et al., 1996; Denley et al., 2008; Engelman et al., 2006; Ruggeri et al., 1998; Samuels et al., 2004; Zhao and Vogt, 2008a; Zhao and Vogt, 2008b). PTEN is a lipid phosphatase that negatively regulates PI3K signaling, and its expression is often lost in cancers, providing yet another method by which PI3K signaling can be deregulated (Li et al., 1997; Maehama and Dixon, 1998; Steck et al., 1997). Additionally, increased activity of upstream regulators can also activate the PI3K signaling pathway, and this can be achieved through amplification or activation of upstream receptor tyrosine kinases or via oncogenic *RAS* mutation (Engelman et al., 2006; Yuan and Cantley, 2008). Interestingly, although *RAS* mutation drives PI3K activity, oncogenic mutations in *RAS* and *PIK3CA* often co-exist in colorectal cancers (Barault et al., 2008; Ollikainen et al., 2007; Velho et al., 2005; Yuan and Cantley, 2008). It is unclear whether these co-existing mutations cooperate to amplify common downstream pathways or function independently to activate non-overlapping pathways (Yuan and Cantley, 2008).

Somatic missense mutations in *PIK3CA* are often observed in tumors of the breast, colon and endometrium. Approximately 80% of mutations map to three hotspots within the coding

sequence of *PIK3CA*: the H1047R mutation is located within the kinase domain, whereas the E542K and E545K mutations are located within the helical domain. These oncogenic mutations result in increased catalytic activity through different mechanisms. The helical domain mutant depends on an interaction with Ras-GTP, whereas the kinase domain mutants depend on an allosteric charge mediated by p85, which mimics Ras-GTP binding (Zhao and Vogt, 2008a; Zhao and Vogt, 2008b; Zhao and Vogt, 2010).

1.3.ii. Therapeutic targeting of the PI3K pathway in cancer

Several lines of experimental evidence suggest that *RAS* mutant tumors depend on the activation of the PI3K pathway. For example, PI3K activity is necessary for transformation of mouse embryonic fibroblasts by oncogenic Ras (Rodriguez-Viciana et al., 1997). In addition, the interaction between Ras and PI3K is essential in a mouse model of Ras-driven tumor formation (Gupta et al., 2007). Collectively these studies demonstrate a requirement for PI3K activity downstream of oncogenic Ras, and suggest that targeting PI3K in *RAS* mutant cancers may have important anti-tumor effects.

Though the aforementioned studies emphasize a role for PI3K signaling in Ras-mediated tumorigenesis, preliminary data suggest that *RAS* mutant tumors are insensitive to single-agent PI3K inhibitors. In fact, *in vitro* experiments have uncovered *RAS* mutation as a dominant predictor of resistance to PI3K inhibitors (Dan et al., 2010; Ihle et al., 2009; Torbett et al., 2008). In addition, murine lung cancers driven by oncogenic K-Ras do not respond to treatment with a single-agent dual PI3K-mTOR inhibitor (Engelman et al., 2008). Therefore, while PI3K activity is an important driver of Ras-mediated transformation and tumorigenesis in cell culture and animal models, inhibition of PI3K pathway activity alone is likely insufficient for the treatment of established tumors harboring *RAS* mutations (Courtney et al., 2010; Engelman, 2009).

The limited response of *RAS* mutant cancer cells to single-agent pathway inhibitors suggests that dual inhibition of MAPK and PI3K signaling may be necessary to block the growth of Ras-

driven tumors. Recent studies show that the efficacy of a single-agent pathway inhibitor is often hindered by the release of negative feedback loops on the reciprocal pathway. For example, treatment of *RAS* mutant cancer cells with potent and specific MEK inhibitors results in increased phosphorylation of the PI3K pathway effector Akt (Hoeflich et al., 2009; Mirzoeva et al., 2009; Sos et al., 2009). The MEK inhibitor-induced activation of Akt signaling was abrogated by co-treatment with an epidermal growth factor receptor (EGFR) inhibitor, suggesting the effect depends on hyperactivation of EGFR. In light of the crosstalk between the MAPK and PI3K signaling pathways, it has been proposed that dual inhibition of both pathways may be required to evade these feedback loops. In support of this hypothesis, several studies demonstrate that combined inhibition of MEK and PI3K signaling in *RAS* mutant cancer cells is superior to single-agent inhibition *in vitro* and *in vivo*, and results in a synergistic decrease in cell viability and increase in apoptosis (Hoeflich et al., 2009; Mirzoeva et al., 2009; Sos et al., 2009). Furthermore, while dual pathway inhibition was also more effective than single-agent pathway inhibition in cancer cells driven by activated receptor tyrosine kinase signaling, the most pronounced synergistic effect was observed in cancer cells harboring oncogenic *RAS* mutations (Sos et al., 2009).

Studies utilizing transgenic mouse models provide additional support for this therapeutic approach. In a mouse model of lung cancer driven by oncogenic K-Ras, single-agent treatment with a dual PI3K-mTOR inhibitor had no effect on tumor growth. Additionally, single-agent treatment with a MEK inhibitor caused only modest tumor regression. However, combined treatment with both pathway inhibitors resulted in synergistic tumor regression (Engelman et al., 2008).

The necessity for dual pathway inhibition is most evident in cancers that harbor co-existing oncogenic mutations in *RAS* and *PIK3CA*. Reports indicate that mutational activation of *PIK3CA* in *KRAS* mutant cancer cells confers resistance to MEK inhibition (Halilovic et al., 2010; Wee et al., 2009). Indeed, treatment with single-agent MEK or Akt inhibitors had no significant effect on tumor growth in a xenograft model with co-existing *KRAS* and *PIK3CA* mutations. However,

combined treatment with both inhibitors was effective at suppressing tumor growth (Halilovic et al., 2010).

Collectively these data indicate that dual inhibition of MAPK and PI3K signaling might be clinically beneficial in *RAS* mutant tumors, and provide a rationale for the design of future clinical trials to test combinations of pathway inhibitors. Further, these studies emphasize that the efficacy of targeted therapeutics is genotype-dependent, and underscore the importance of stratifying patients by tumor genotype prior to therapy.

1.4. NEGATIVE FEEDBACK REGULATION OF RAS SIGNALING

The intensity and duration of Ras signaling is carefully controlled by a number of negative feedback mechanisms. The receptor tyrosine kinases EGFR and EphA2 are both involved in negative feedback loops mediated by high Ras/MAPK pathway activity, and will be discussed in further detail below.

1.4.i. Epidermal growth factor receptor signaling

The epidermal growth factor receptor family consists of EGFR/ErbB1, ErbB2, ErbB3 and ErbB4 (Hynes and Lane, 2005). All members contain an extracellular region (domains I, II, III and IV), followed by a single membrane spanning region and cytoplasmic tyrosine kinase domain. Ligand binding to ErbB family members induces the formation of homodimers and heterodimers and subsequent activation of tyrosine kinase activity. Ligand binding to domains I and III of the extracellular region of the receptor induces a conformational change that unmasks a previously occluded receptor dimerization arm in domain II, allowing for dimerization with another ligand-bound receptor (Lemmon and Schlessinger, 2010). This results in the subsequent activation of receptor tyrosine kinase activity and autophosphorylation of the cytoplasmic tails of each dimer

pair, thereby creating docking sites for several intracellular signaling and adaptor proteins (Hynes and Lane, 2005; Lemmon and Schlessinger, 2010; Wheeler et al., 2010). The MAPK and PI3K signaling cascades are two of the main pathways initiated upon ErbB receptor activation (Hynes and Lane, 2005).

ErbB3 has impaired tyrosine kinase activity due to substitutions in critical amino acid residues in the tyrosine kinase domain of the receptor. However, ligand-induced heterodimerization with other ErbB family members results in the phosphorylation of tyrosine residues in the intracellular portion of the ErbB3 receptor, which serve as docking sites for several signaling and adaptor proteins. (Wheeler et al., 2010). ErbB3 contains six docking sites for the p85 subunit of PI3K and very efficiently activates the PI3K pathway (Hynes and Lane, 2005).

The ErbB family of ligands can be divided into three groups. The first group includes epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and amphiregulin, which bind specifically to EGFR. The second group includes betacellulin, heparin-binding EGF (HB-EGF) and epiregulin, which bind both EGFR and ErbB4. The third group is comprised of neuregulins (NRGs), which bind ErbB3 (NRG1, NRG2) and ErbB4 (NRG1, NRG2, NRG3, NRG4). ErbB2 has no known ligands, but is the preferred heterodimerization partner of the other ErbB family members (Hynes and Lane, 2005).

A number of phosphorylation events mediated by activated ERK serve to negatively regulate EGFR and subsequent downstream signaling to the MAPK and PI3K pathways. Phosphorylation of Raf-1 by ERK disrupts the Ras/Raf interaction, desensitizes Raf-1 to mitogenic stimulation and reduces Raf-1 kinase activity (Dougherty et al., 2005). Direct phosphorylation of SOS1 by ERK disrupts the interaction between SOS1 and Grb2, thereby preventing membrane recruitment of SOS1 and subsequent Ras activation (Porfiri and McCormick, 1996). In addition, phosphorylation of the adaptor protein Gab1 by ERK prevents the recruitment and activation of PI3K to EGFR (Yu et al., 2002).

ERK also directly phosphorylates a threonine residue (T669) located within the juxtamembrane region of EGFR to inactivate the receptor, although the exact mechanism by which this phosphorylation alters receptor activity is not completely understood (Lemmon and Schlessinger, 2010; Li et al., 2008). This phosphorylation site may be involved in EGFR turnover and trafficking, as ERK-mediated phosphorylation at this site slows the pace of EGF-induced receptor down-regulation (Gan et al., 2010; Li et al., 2008). In an *NRAS* mutant fibrosarcoma cell line, EGFR is constitutively phosphorylated at T669 due to high levels of basal ERK signaling. Pharmacological inhibition of MEK eliminates the EGF-induced phosphorylation of T669 and potentiates the EGF-induced phosphorylation of EGFR at several activating tyrosine residues (including Y1068 and Y1045), resulting in increased acute activation of EGFR and Akt signaling (Gan et al., 2010). The observation that MEK inhibition relieves a negative feedback loop to the PI3K pathway that is mediated by EGFR has been made by several other groups as well (Hoeflich et al., 2009; Mirzoeva et al., 2009; Sos et al., 2009; Yoon et al., 2009).

Dual pharmacological inhibition of MEK and EGFR may be required to evade the feedback loops unleashed by single-agent MEK inhibition. Studies utilizing phospho-protein arrays show that treatment with MEK inhibitors increases the phosphorylation of EGFR, ErbB2, ErbB3 and Akt (Mirzoeva et al., 2009; Yoon et al., 2009; Yoon et al., 2010). *KRAS* mutant cancer cells are resistant to single-agent EGFR inhibitor treatment and have variable responses to single-agent MEK inhibitor treatment. However, dual treatment with MEK and EGFR inhibitors suppresses the compensatory activation of receptor tyrosine kinase and Akt signaling induced by single-agent MEK inhibition, and also significantly reduces the IC_{50} of the EGFR inhibitor (Yoon et al., 2009; Yoon et al., 2010). Accordingly, combined treatment with small molecule inhibitors of EGFR and MEK (gefitinib and AZD6244, respectively) synergistically inhibits *KRAS* mutant gastric cancer and lung cancer cell growth and enhances apoptosis *in vitro* and *in vivo* (Yoon et al., 2009; Yoon et al., 2010).

Recent studies demonstrate that silencing oncogenic *KRAS* expression may also sensitize cells to EGFR inhibitor treatment. These studies were prompted by the observation that small hairpin

RNA (shRNA)-mediated depletion of oncogenic K-Ras expression partially inhibited the growth of NSCLC cancer cells *in vitro* and *in vivo*, but did not result in the induction of cell death (Sunaga et al., 2011). Although the levels of MEK and ERK phosphorylation were reduced by shRNA-mediated depletion of K-Ras, the levels of STAT, EGFR and Akt phosphorylation were actually increased (Sunaga et al., 2011). Due to the release of these negative feedback loops, shRNA-mediated depletion of oncogenic K-Ras sensitized cells to treatment with either p38 or EGFR inhibitors (Sunaga et al., 2011). These results suggest that targeting oncogenic K-Ras alone may be inefficient due to the compensatory activation of other signaling pathways. Importantly, these findings complement observations made using MEK inhibitors: a reduction in MAPK signaling - whether by silencing oncogenic *KRAS* expression or by pharmacological inhibition of MEK activity - results in the compensatory activation of receptor tyrosine kinases and increased Akt signaling.

Evidence has emerged that oncogenic K-Ras alters EGFR localization, resulting in reduced EGF binding and internalization (van Houdt et al., 2010). In cancer cell lines with oncogenic *KRAS* mutations, EGFR is localized predominantly in intracellular vesicles; this localization is not significantly altered after stimulation with EGF. Loss of oncogenic *KRAS* expression, by either RNA interference or by deletion via homologous recombination, restores plasma membrane localization of EGFR as well as proper internalization of the receptor upon stimulation with EGF. Accordingly, loss of oncogenic *KRAS* expression sensitized cells to EGF-induced activation of EGFR signaling. In addition, loss of oncogenic *KRAS* expression also sensitized cells to EGFR inhibitors. In a panel of human colorectal carcinomas, oncogenic *KRAS* mutation appeared to be associated with loss of normal basolateral EGFR localization, which likely influences the responsiveness of tumors to EGFR ligands and inhibitors (van Houdt et al., 2010).

Clinical studies demonstrate that oncogenic *KRAS* mutations are predictive of resistance to EGFR-based therapies (Wheeler et al., 2010). In light of the studies discussed above, it appears that oncogenic mutation in *KRAS* desensitizes cells to EGFR inhibitors by at least two mechanisms. The first and most intuitive explanation is that oncogenic mutation in *KRAS* results in a constitutive signal to effector pathways downstream of EGFR, such that activation or

inhibition of EGFR signaling would have little to no effect on pathways downstream of K-Ras. The studies discussed above present an additional novel mechanism by which oncogenic mutation in *KRAS* desensitizes cells to EGFR inhibitors, involving a complete rewiring of EGFR signaling dynamics. Though the molecular mechanism is not fully understood, it appears that oncogenic K-Ras desensitizes cells to EGFR activation and inhibition by altering EGFR trafficking, turnover and localization in a MEK-dependent fashion (Gan et al., 2010; Li et al., 2008; Sunaga et al., 2011; van Houdt et al., 2010; Yoon et al., 2009; Yoon et al., 2010). Suppression of MEK signaling – whether by RNAi-mediated depletion of oncogenic K-Ras expression or by pharmacological inhibition of MEK kinase activity – resensitizes EGFR signaling and primes the receptor for activation or inhibition. Accordingly, treatment with a MEK inhibitor alongside an EGFR inhibitor synergistically inhibits the growth of cancer cells harboring oncogenic *KRAS* mutations and significantly reduces the IC_{50} for the EGFR inhibitor (Yoon et al., 2009; Yoon et al., 2010). Taken together, these data suggest dual targeting of EGFR and the MAPK signaling pathway might be beneficial in cancers harboring oncogenic *KRAS* mutations.

1.4.ii. Eph receptor tyrosine kinases

In addition to the core components of the MAPK cascade, there are a number of negative modulators that act to regulate the intensity and duration of signaling downstream of Ras. Several endogenous antagonists of Ras-mediated signaling have been identified, including Sprouty and Spred proteins as well as dual specificity phosphatases (DUSPs) (Murphy et al., 2010). Perhaps unsurprisingly, high levels of receptor tyrosine kinase signaling and/or MAPK pathway activation induce the expression of many of these regulatory proteins as a means of negative feedback.

Several years ago, our laboratory found that expression of a receptor tyrosine kinase called EphA2 is induced by high MAPK pathway activity. Furthermore, we found that stimulation of EphA2 with its ligand ephrin-A1 attenuates ERK phosphorylation, thereby creating a negative feedback loop to regulate MAPK activity (Macrae et al., 2005). Other laboratories have also

made similar observations regarding the ability of activated EphA2 to negatively regulate the Ras/MAPK pathway (Miao et al., 2001). Thus, EphA2 represents yet another MAPK pathway target involved in the negative feedback control of Ras/MAPK signaling. The crosstalk between the Ras/MAPK pathway and signaling downstream of the activated EphA2 receptor will be discussed in further detail in Chapter 3.

1.5. GROWTH SUPPRESSIVE AND ANTAGONISTIC PROPERTIES OF WILD-TYPE RAS

It has long been thought that oncogenic Ras acts in a dominant fashion over wild-type Ras, and this is supported by the observation that an oncogenic *RAS* allele can transform cells even if a wild-type *RAS* allele is expressed. However, several studies suggest that the relative overexpression of the oncogenic *RAS* allele is required to confer dominance over the wild-type allele. An early study demonstrated this by utilizing homologous recombination to replace one allele of wild-type *Hras1* with oncogenic *Hras1* in Rat-1 cells (Finney and Bishop, 1993). The resultant heterozygous cells expressed wild-type and oncogenic *Hras1* at equivalent levels, but were not transformed. Over time, spontaneously transformed cells arose from the culture of heterozygous cells. Importantly, the transformed cells had amplified the mutant allele, suggesting the transforming capability of oncogenic *Hras1* depends on its relative overexpression with respect to wild-type *Hras1* (Finney and Bishop, 1993). These studies have led to a deeper investigation into the role of wild-type Ras signaling in the context of an oncogenic *RAS* mutation.

1.5.i. Human tumor samples and mouse models

Several studies report that expression of the wild-type *RAS* allele is lost in tumors harboring oncogenic *RAS* mutations. Chemically-induced murine thymic lymphomas, skin and lung tumors harboring oncogenic mutations in *Nras*, *Hras1* and *Kras2*, respectively, all exhibit allelic loss of the corresponding wild-type allele (Bremner and Balmain, 1990; Buchmann et al., 1991; Guerrero

et al., 1985; Hegi et al., 1994). In addition, human lung tumors harboring oncogenic *KRAS* mutations show loss of heterozygosity at the *KRAS* genetic locus (Li et al., 2003). Taken together, these studies suggest that there is strong selective pressure to lose expression of the wild-type *RAS* allele.

A recent study provides evidence that wild-type Ras exhibits tumor suppressive properties *in vitro* and *in vivo* (Zhang et al., 2001). Treatment of wild-type and *Kras2* heterozygous transgenic mice with two different carcinogens produced lung tumors bearing oncogenic *Kras*2 mutations. Mice that initially carried only one wild-type copy of *Kras2* developed many more tumors than mice that initially carried two wild-type copies of the gene. Additionally, the tumors that developed in the *Kras2* heterozygous mice were much larger and less differentiated. Therefore, these studies implicate wild-type *Kras2* as a tumor suppressor in chemically-induced lung carcinogenesis (Zhang et al., 2001). Furthermore, *in vitro* studies demonstrate that ectopic expression of wild-type Kras2 inhibits the growth of cell lines harboring oncogenic *Kras2* mutations in a dose-dependent manner. In many of the resulting clones, there was an inverse correlation between the expression of ectopic wild-type Kras2 and ERK phosphorylation levels, suggesting that wild-type Kras2 might antagonize oncogenic Kras2-driven MAPK signaling. However, in many clones, cell growth was inhibited without any significant reduction in ERK phosphorylation, suggesting wild-type Kras2 can inhibit cell growth through additional mechanisms as well. Finally, an analysis of mouse lung tumors revealed a correlation between *Kras2* mutation and loss of the corresponding wild-type allele. These studies clearly demonstrate that wild-type Ras can antagonize the transforming potential of its oncogenic counterpart in a dose-dependent manner, although the mechanism by which it does so is not well understood. Importantly, this work reveals a surprising role for wild-type Ras as a tumor suppressor in the context of oncogenic Ras, and suggests the ratio between wild-type and oncogenic *RAS* expression is an important determinant of transforming potential.

The study discussed above describes a tumor suppressive role for the corresponding wild-type allele of the oncogenic *RAS* isoform. However, recent work also indicates that oncogenic Ras

activity can be antagonized by other canonical wild-type Ras isoforms as well (To et al., 2008). Two different strains of knock-in transgenic mice were used in the study. The *Hras* knock-in ($Hras^{KI}$) mouse does not express endogenous *Kras*, but instead carries a "knock-in" *Hras* cDNA expressed from the *Kras* genetic locus. The *Kras* knock-in ($Kras^{KI}$) strain, in which the K-Ras 4B cDNA was knocked back into the *Kras* locus, was used as a control. $Kras^{LA2}$ mice, which undergo spontaneous recombination to activate *Kras*, were bred to wild-type mice as well as to heterozygous mice from each of the knock-in strains. The resulting animals harbored one $Kras^{LA2}$ allele and one wild-type *Kras* or knock-in ($Hras^{KI}$ or $Kras^{KI}$) allele. Mice with one $Kras^{KI}$ allele, which is capable of producing only K-Ras 4B, developed approximately three times as many tumors as mice harboring the endogenous wild-type *Kras* allele, which can produce both K-Ras 4A and K-Ras 4B. The results suggest that the tumor suppressive function of wild-type *Kras* involves the 4A isoform. Importantly, the $Hras^{KI}$ allele was able to inhibit tumor development as efficiently as the wild-type *Kras* allele, suggesting that the inhibitory function of wild-type Ras is not isoform specific.

1.5.ii. Synthetic dominant negative Ras mutants

The mechanism by which wild-type Ras antagonizes the transforming capacity of oncogenic Ras is not well understood. It is plausible that the predominantly GDP-bound wild-type Ras has a unique set of effectors and promotes its own growth inhibitory signaling pathways, although effectors of Ras-GDP have not been identified. Alternatively, wild-type Ras might compete with oncogenic Ras for regulators and/or effectors in a co-dominant fashion (Pfeifer, 2001; Singh et al., 2005). These questions can be addressed by utilizing synthetic Ras mutants that are constitutively GDP-bound.

The dominant negative Ras synthetic mutant contains a serine to asparagine conversion at position 17, resulting in a preferential affinity for GDP. Because Ras N17 is constitutively GDP-bound, it forms inactive complexes with exchange factors and prevents nucleotide loading of

endogenous Ras proteins. Importantly, Ras N17 selectively blocks nucleotide loading of wild-type, but not oncogenic, Ras isoforms (Stewart and Guan, 2000).

Reports indicate that ectopic expression of Ras N17 inhibits cell growth and that this effect can be overcome by overexpression of oncogenic Ras (Feig and Cooper, 1988). Notably, oncogenic Ras antagonizes the growth inhibitory activity of Ras N17 only when it is co-expressed in excess. Conversely, transformation of NIH3T3 cells by oncogenic Ras is inhibited when Ras N17 is co-expressed at an excess of oncogenic Ras (Feig and Cooper, 1988; Stewart and Guan, 2000). These data indicate that the relative levels of Ras N17 and oncogenic Ras are important in determining phenotypic outcome, and suggest that Ras-GDP and Ras-GTP might act in a co-dominant fashion.

It is not exactly clear how synthetic Ras mutants inhibit the transforming potential of oncogenic Ras. As discussed above, it is plausible that Ras N17 (or wild-type Ras-GDP) antagonizes oncogenic Ras signaling by sustaining unique and independent growth-inhibitory signaling pathways. Alternatively, Ras N17 (or wild-type Ras-GDP) might compete with oncogenic Ras (or wild-type Ras-GTP) for regulators, effectors or proper localization. Another interpretation is that Ras N17 prevents the activation of endogenous wild-type Ras isoforms, and that despite the presence of oncogenic Ras, this is sufficient to inhibit cell growth. Future experiments in which Ras N17 and oncogenic Ras are expressed at equivalent levels, or in which Ras N17 is expressed in human cancer cell lines that endogenously express oncogenic Ras, might help address some of these issues.

1.5.iii. Membrane-targeted p120 RasGAP

Another approach to study the role of wild-type Ras signaling is to utilize an artificially membrane-targeted form of p120 RasGAP to constitutively promote the conversion of endogenous Ras-GTP to Ras-GDP. p120 RasGAP is comprised of an N-terminal regulatory region and a C-terminal catalytic region, and is predominantly localized in the cytosol. The N-terminal regulatory region

contains two Src homology 2 (SH2) domains flanking a Src homology 3 (SH3) domain, which facilitate the recruitment of p120 RasGAP to adaptor and signaling proteins located at the plasma membrane, including activated receptor tyrosine kinases. The C-terminal catalytic region contains the conserved GAP-related domain (GRD), which binds activated Ras and stimulates GTP hydrolysis (Pamonsinlapatham et al., 2009).

The C-terminal portion of K-Ras 4B contains a CAAX motif and a polybasic domain which undergo post-translational modification to firmly anchor K-Ras 4B in the plasma membrane. The addition of the last 19 amino acids of K-Ras 4B, which contain these plasma membrane localization signals, to the C-terminus of p120 RasGAP is sufficient to artificially target p120 RasGAP to the plasma membrane (Clark et al., 1993; Huang et al., 1993). Membrane-targeted p120 RasGAP is a potent suppressor of cell growth, and its growth inhibitory effect requires catalytic activity (Huang et al., 1993). These observations are consistent with the idea that membrane-targeted p120 RasGAP suppresses growth via constitutive down-regulation of endogenous Ras activity.

Overexpression of oncogenic Ras blocks the growth inhibitory properties of membrane-targeted p120 RasGAP in NIH3T3 cells. However, the majority of clones that grow as a result of co-expression of oncogenic Ras have an untransformed phenotype, indicating membrane-targeted p120 RasGAP can suppress oncogenic Ras-mediated transformation (Clark et al., 1993; Huang et al., 1993). Interestingly, in clones that display a transformed phenotype, oncogenic Ras is expressed at a much higher level than membrane-targeted p120 RasGAP (Huang et al., 1993). In addition, membrane-targeted p120 RasGAP suppresses the ability of oncogenic Ras to induce foci formation in NIH3T3 cells in a dose-dependent manner. Importantly, a catalytic inactive mutant of membrane-targeted p120 RasGAP is unable to block oncogenic Ras-driven foci formation (Huang et al., 1993).

The mechanism by which membrane-targeted p120 RasGAP suppresses transformation by oncogenic Ras is not clear. Membrane-targeted p120 RasGAP constitutively down-regulates the

activity of endogenous wild-type Ras; oncogenic Ras is resistant to GAP-mediated hydrolysis. It is possible that membrane-targeted p120 RasGAP might bind oncogenic Ras and form signaling incompetent complexes; however, if this was the case, the catalytic inactive mutant of membrane-targeted p120 RasGAP should also be able to antagonize the transforming potential of oncogenic Ras. One interpretation is that transformation results from additive signals from both oncogenic and wild-type Ras isoforms, and that the constitutive down-regulation of endogenous wild-type Ras activity by membrane-targeted p120 RasGAP is sufficient to inhibit transformation by oncogenic Ras. Alternatively, it may be that wild-type Ras, in a GDP-bound form, directly antagonizes oncogenic Ras activity. In any case, the results obtained from experiments utilizing membrane-targeted p120 RasGAP are consistent with studies described earlier suggesting that wild-type Ras exerts growth suppressive properties and antagonizes transformation by oncogenic Ras.

1.6. AIMS OF THIS STUDY

Mutation in one of three canonical Ras genes - *HRAS*, *KRAS* or *NRAS* - is observed in an estimated 30% of human cancers. The oncogenic Ras isoform is thought to be primarily responsible for mediating the activation of downstream effector pathways, although the two wild-type Ras isoforms remain functional. In fact, the signaling contribution of the wild-type Ras isoforms in this context has largely been discounted and unexamined.

This study aims to determine whether wild-type Ras contributes to the activation of MAPK and PI3K signaling in cancer cells with oncogenic *RAS* mutations. The ability of intrinsic negative regulators (EphA2) and activators (EGFR) to modulate wild-type and oncogenic Ras signaling will be investigated in detail. In addition, the contribution of wild-type and oncogenic Ras to basal steady-state levels of MAPK and PI3K signaling will also be examined. The results of this work will uncover interesting aspects of wild-type Ras biology that have been previously underappreciated.

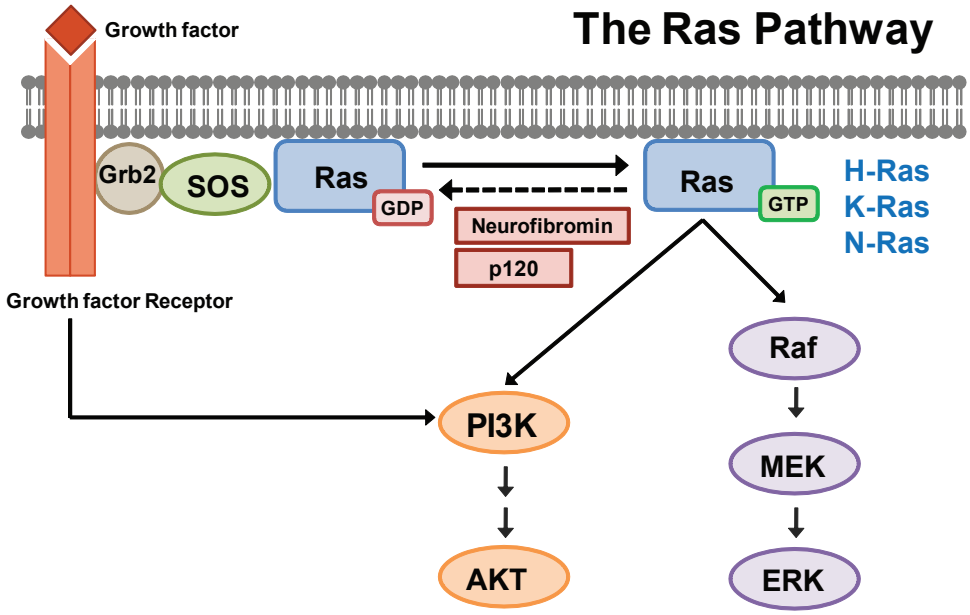

Figure 1.1. The Ras Pathway

Ras is a small G protein that cycles between an inactive GDP-bound form and an active GTP-bound form. When growth factor receptors at the cell surface are activated, guanine nucleotide exchange factors such as SOS promote the exchange of GDP for GTP on Ras, thereby turning on Ras signaling. In contrast, GTPase activating proteins, including neurofibromin and p120 RasGAP, accelerate the hydrolysis of Ras-GTP to Ras-GDP, thereby maintaining a steady state of Ras signaling. In its active GTP-bound form, Ras interacts with various downstream effector proteins to turn on a number of downstream signaling pathways, including the MAPK and PI3K signaling pathways, depicted here.

CHAPTER 2.

MATERIALS AND METHODS

2.1. REAGENTS

Recombinant ephrin-A1-Fc (EA1-Fc) and ephrin-B1-Fc (EB1-Fc) (R&D Systems, Catalog # 602-A1 and 473-EB, respectively) were resuspended in PBS at a concentration of 200 ng/ul (200x) and stored at -80°C. Human IgG Fc fragment (Jackson ImmunoResearch, Catalog # 009-000-008) was resuspended in PBS at a concentration of 200 ng/ul and stored at -80°C.

Recombinant human EGF (Invitrogen, Catalog # PHG0311) was resuspended in PBS at a concentration of 100-125 ng/ul and stored and -80°C; a working stock solution was obtained by diluting the original stock solution with PBS at a final working concentration of 1 ng/ul.

The MEK inhibitor U0126 was dissolved in dimethyl sulfoxide (DMSO) to create a 10 mM stock solution (1000x) and was stored at -20°C.

2.2. CONSTRUCTS

All Ras constructs were of human origin and have been described previously (Rodriguez-Viciana et al., 2004). Genes were cloned into pENTR or pDONR 221 vectors (Invitrogen) and transferred in frame into cytomegalovirus promoter-driven expression plasmids with N-terminal myc-tags, or into a Gateway-compatible derivative of the pFBNeo retroviral vector, using recombination-mediated Gateway technology (Invitrogen). Mutations were introduced by site-directed-mutagenesis.

2.3. ANTIBODIES

The primary antibodies used in this study are listed below in Table 2.1.

Table 2.1. Commercial primary antibodies

ANTIBODY	SOURCE	COMPANY	CATALOG NUMBER
Akt	mouse	Millipore	05-591
c-Myc	rabbit	Santa Cruz Biotechnology	sc-789
cyclin D1	mouse	Santa Cruz Biotechnology	sc-8396
EGFR	rabbit	Santa Cruz Biotechnology	sc-03
EGFR	mouse	Cell Signaling Technology	2239
EphA2	rabbit	Santa Cruz Biotechnology	sc-924
EphB2	mouse	R&D Systems	AF467
GFP	rabbit	Santa Cruz Biotechnology	sc-8334
H-Ras	rabbit	Santa Cruz Biotechnology	sc-520
K-Ras	mouse	Santa Cruz Biotechnology	sc-30
MEK ½	rabbit	Cell Signaling Technology	9122
Neurofibromin	rabbit	Santa Cruz Biotechnology	sc-67
N-Ras	mouse	Santa Cruz Biotechnology	sc-31
p27	rabbit	Santa Cruz Biotechnology	sc-528
p44/42 MAPK	rabbit	Cell Signaling Technology	9102
pan-Ras	mouse	BD Biosciences	610002
Phospho-Akt Ser473	rabbit	Cell Signaling Technology	4058
Phospho-Akt Thr308	rabbit	Cell Signaling Technology	9275
Phospho-EGFR Thr669	rabbit	Cell Signaling Technology	3056
Phospho-EGFR Tyr1068	rabbit	Cell Signaling Technology	4407
Phospho-EGFR Tyr1173	rabbit	Cell Signaling Technology	4407
Phospho-MEK 1/2 Ser217/Ser221	rabbit	Cell Signaling Technology	9121
Phospho-p44/42 MAPK Thr202/Tyr204	mouse	Cell Signaling Technology	9106
Phospho-Raf-1 Ser338	rabbit	Cell Signaling Technology	9427
Phospho-Tyrosine	mouse	Santa Cruz Biotechnology	sc-508
Raf-1	mouse	BD Transduction	610152
Ras GAP	mouse	Millipore	05-178

2.4. CELL LINES AND CULTURE CONDITIONS

Cancer cell line origin, mutational status and concentration of EGF used for acute stimulation are described below in Table 2.2.

Table 2.2. Cancer cell lines

Cancer cell line	Origin	MAPK/PI3K pathway mutation*	EGF (ng/ml)
A549	Lung cancer	*KRAS* G12S	10
ACCS	Bladder cancer	*HRAS* G12V	20
BT549	Breast cancer	*PTEN 822delG (L295X)*	30
HBL100	Breast cancer	None reported/detected	10-20
HCT-116	Colon cancer	*PIK3CA* H1047R; *KRAS* G13D (heterozygous)	30
MDA-MB-231	Breast cancer	*BRAF* G464V; *KRAS* G13D (heterozygous)	30
MIA PaCa-2	Pancreatic cancer	*KRAS* G12C	30
Panc1	Pancreatic cancer	*KRAS* G12D (heterozygous)	20
PC3	Prostate cancer	*PTEN* homozygous deletion	30
RD	Rhabdomyosarcoma	*NRAS* Q61H	30
ST88	Malignant peripheral nerve sheath tumor (MPNST)	*NF-/-*	20
WM278	Melanoma	*BRAF* V600E	20

* Mutations in *RAS* are homozygous unless otherwise indicated.

All cells were grown at 37°C in a 5% CO_2 incubator. PC3 cells were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum. All other cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. $RasGAP^{+/+}$ MEFs (Clone 12.78) and $RasGAP^{-/-}$ MEFs (Clone 12.64) were a kind gift from Dr. Tony Pawson. The ACCS cell line is a derivative of the T-24 bladder cancer cell line (Phuchareon et al., 2009), and was a kind gift from Dr. Osamu Tetsu. HBL100 cells stably expressing the ecotropic receptor were a kind gift from Dr. Pablo Rodriguez-Viciana.

2.5. TRANSIENT TRANSFECTIONS

HEK 293 cells were seeded in 6-well plates at 10^6 cells per well and transfected the following day with 2 µg total plasmid DNA and 5 µl of Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The following day, cells were placed in serum-free media and starved overnight. The following day (48 hours after transfection), cells were stimulated with EGF and lysed for immunoblot analysis.

2.6. NUCLEOFECTION

DNA was introduced into RasGAP $^{+/+}$ and RasGAP $^{-/-}$ MEFs using the Nucleofector II Device (Lonza AG) according to manufacturer's instructions. Briefly, 2 x 10^6 cells were resuspended in 100 µl MEF Nucleofector Kit 2 solution (Lonza AG) per nucleofection and subjected to nucleofection program T-020. Cells were harvested 24 - 48 hours post nucleofection.

2.7. GENERATION OF STABLE CELL LINES

Phoenix-Eco packaging cells were transfected with pFBNeo retroviral vectors using Lipofectamine 2000 (Invitrogen). Supernatants containing packaged retroviral particles were harvested 48 and 72 hours after transfection, filtered through 0.45 µm filters, supplemented with 5 µg/ml polybrene, and incubated with plated HBL100-EcoR cells overnight at 37°C. Infected cells were then cultured in growth medium supplemented with 1 mg/ml G418 (Invitrogen) to select for transduced cells.

2.8. RNA INTERFERENCE

One day prior to transfection, cells were seeded in 6-well plates at 2-3 x 10^5 cells per well. Cells were transfected with 80 nM siRNA (Qiagen) twice in 24 hour intervals using Lipofectamine RNAiMAX (Invitrogen). The day after the second siRNA transfection (48 hours after the initial transfection), cells were placed in serum-free media and starved overnight. Cells were then subjected to EGF stimulation experiments the following day (72 hours after the initial transfection).

For ephrin stimulation experiments, cells were seeded in 6-well plates in duplicate at 4 x 10^5 cells per well one day prior to transfection. Cells were transfected with 80 nM siRNA (Qiagen) twice in 24 hour intervals using Lipofectamine RNAiMAX (Invitrogen). The day after the second siRNA transfection (48 hours after the initial transfection), cells were trypsinized, duplicate wells were pooled, and cells were counted and seeded at 2-3 x 10^5 cells per well in 6-well plates. The following day (72 hours after the initial transfection), cells were placed in serum-free media and starved overnight. Cells were subjected to ephrin stimulation experiments the following day (96 hours after the initial transfection).

For cell proliferation analysis experiments, cells were seeded in 6-well plates in duplicate at 3-5 x 10^5 cells per well one day prior to transfection. Cells were transfected with 80 nM siRNA (Qiagen) twice in 24 hour intervals using Lipofectamine RNAiMAX (Invitrogen). The day after the second siRNA transfection (48 hours after the initial transfection), cells were trypsinized, duplicate wells were pooled, and cells were counted and seeded at equal densities for cell proliferation analysis (see section 2.13 for detailed methods).

Sequences for all commercial siRNAs are provided below in Table 2.3.

Table 2.3. siRNA sequences

siRNA name	Qiagen product name	Catalog number	Sequence
Non-silencing (NS)	AllStars Negative Control	1027281	proprietary
HRAS (H1)	Hs_HRAS_6	SI02654806	5'-CCGGAAGCAGGTGGTCATTGA-3'
H2	Hs_HRAS_7	SI02662030	5'-CACAGATGGGATCACAGTAAA-3'
H3	Hs_HRAS_9	SI03068989	5'-CAGGAAGGAGGTGCAGACGGA-3'
KRAS (K1)	Hs_KRAS_1	SI03101903	5'-GACGATACAGCTAATTCAGAA-3'
K2	Hs_KRAS2_3	SI00071015	5'-CAGACGTATATTGTATCATTT-3'
K3	Hs_KRAS2_8	SI02662051	5'-AAGGAGAATTTAATAAAGATA-3'
NRAS (N1)	Hs_NRAS_3	SI00038899	5'-CCAGCTCTCAGTAGTTTAGTA-3'
N2	Hs_NRAS_5	SI00300993	5'-AACCTGTTTGTTGGACATACT-3'
N3	Hs_NRAS_6	SI02662632	5'-CTGAGATACGTCTGTGACTTA-3'
p120 (p120-1)	Mm_Rasa1_3_HP	SI00236915	5'-GAAGAGTACAGTGAATTTAAA-3'
p120-2	Hs_RASA1_3_HP	SI00045360	5'-ACGGACCTGTCCCGTGATTTA-3'

2.9. EPHRIN STIMULATION EXPERIMENTS

Cells were seeded in 6-well plates at 2-3 x 10^5 cells per well, serum starved overnight, and pretreated with 1 µg/ml ephrin-A1-Fc (EA1-Fc) for 5 minutes. In some cases, after ephrin pretreatment cells were stimulated with a growth factor such as EGF to induce robust activation of MAPK and PI3K signaling.

Pre-clustered ephrin-B1-Fc ligand was generated by incubation with human IgG Fc fragment at a fixed ratio of 0.1x the concentration of ephrin-B1-Fc for 1 hour at 4°C, as described by Stein and colleagues (Stein et al., 1998). Cells were seeded in 6-well plates at 2-3 x 10^5 cells per well, serum starved overnight, and pretreated with 2 µg/ml pre-clustered ephrin-B1-Fc (EB1-Fc) for 10 minutes. In some cases, after ephrin pretreatment cells were stimulated with a growth factor such as EGF to induce robust activation of MAPK and PI3K signaling.

2.10. PROTEIN EXTRACTION

Cells were washed twice in ice cold PBS and lysed in 1% Triton lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na_2VO_4, and 1 mM DTT) supplemented with a protease inhibitor cocktail (Complete Mini, Roche) unless they were to be subjected to a Ras-GTP assay, in which case they were lysed in 1% TX100-TNM lysis buffer (20 mM Tris pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl,1% Triton-X100) supplemented with 1 mM DTT, a protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails (Sigma). After addition of lysis buffer, samples were rocked at 4°C for 10 – 30 minutes, scraped, collected and cleared by centrifugation at 13,000 rpm for 10 minutes at 4°C. Protein levels were quantified by the Bio-Rad Protein Assay (Bio-Rad), normalized to equal concentrations and boiled in LDS sample buffer (Invitrogen) supplemented with DTT for 10 minutes at 70°C.

2.11. RAS-GTP ASSAY

Cells were washed twice in ice cold PBS and lysed in 1% TX100-TNM lysis buffer (20 mM Tris pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl,1% Triton-X100) supplemented with 1 mM DTT, a protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails (Sigma). Samples were rocked at 4°C for 10 – 30 minutes, scraped, collected and cleared by centrifugation at 13,000 rpm for 10 minutes at 4°C. Protein levels were quantified by the Bio-Rad Protein Assay (Bio-Rad), and normalized to equal concentrations. A portion of each sample (45 – 60 µl) was added to LDS sample buffer (Invitrogen) supplemented with DTT and boiled at 70°C for 10 minutes for immunoblot analysis of total protein concentrations. Equal amounts of protein from each sample were also added to 10 µl of packed GST-Raf-RBD beads in 300-500 µl of 1% TX100-TNM lysis buffer supplemented with DTT, protease and phosphatase inhibitors, and rotated at 4°C for 1-2 hours. Beads were washed three times with 1 ml of cold lysis buffer, drained with a 26-gauge needle, and boiled in LDS sample buffer supplemented with DTT for 10 minutes at 70°C.

2.12. IMMUNOBLOT ANALYSIS

Equal amounts of protein extracts were resolved using 4-12% Bis-Tris or 3-8% Tris-Acetate SDS-polyacrylamide gel electrophoresis (NuPAGE, Invitrogen) and transferred to a nitrocellulose membrane. After blocking for at least 1 hour at room temperature with TBST (Tris-Buffered Saline and Tween 20) supplemented with 5% dry milk, membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies were detected with secondary antibodies labeled with either IRDye800 (Rockland), Alexa Fluor 680 (Molecular Probes) or horseradish peroxidase (HRP)-conjugated secondary antibodies. Fluorescent antibodies were visualized using a LI-COR-Odyssey scanner and HRP antibodies were visualized with chemiluminescence followed by autoradiography.

2.13. CELL PROLIFERATION ANALYSIS

For serum starvation experiments, 4-6 × 10^3 cells were seeded in triplicate in 12-well plates, and accurate cell counts were obtained using a Coulter particle analyzer. Cell counts were obtained 24 hours after seeding as well as 72 hours after serum starvation. Time zero was taken 24 hours after cell seeding, at the time of serum starvation.

For siRNA experiments, cells were seeded in 6-well plates in duplicate at 3 - 5 x 10^5 cells per well one day prior to transfection. Cells were transfected with 80 nM siRNA (Qiagen) twice in 24 hour intervals using Lipofectamine RNAiMAX (Invitrogen). The day after the second siRNA transfection (48 hours after the initial transfection), cells were trypsinized, duplicate wells were pooled, and cells were counted and seeded in 12-well plates in triplicate at 4 - 6 x 10^3 cells per well for cell proliferation analysis. Cells were also seeded in 6-well plates at 1 - 1.5 x 10^5 cells per well for immunoblot analysis. Samples were harvested for immunoblot analysis and cell proliferation analysis every 24 hours for a 5-day period. Time zero (48 hours after initial siRNA transfection) was taken immediately at the time of seeding. Accurate cell counts were obtained using a Coulter particle analyser.

2.14. QUANTITATIVE REAL TIME PCR (qRT-PCR)

RNA was extracted using the RNase Easy kit (Qiagen) and DNase-treated. RNA was reverse-transcribed into cDNA. Quantitative PCR analysis was performed on all samples in triplicate using an AB Prism 7900 sequence detection system. All samples were normalized to *H.Gus* expression. DNase treatment, reverse transcription, qRT-PCR and data analysis were performed by the UCSF Helen Diller Family Comprehensive Cancer Center Genome Analysis Core. Tables 2.4, 2.5, and 2.6 contain information regarding assay reagents.

Table 2.4. qRT-PCR reagents for *HRAS* assay

Assay component	Sequence
Forward primer	GGAACAAGTGTGACCTGGCTG
Reverse primer	AACGTGTAGAAGGCATCCTCC
Probe	AATCTCGGCAGGCTCAGGACCTCG
Amplicon	GGAACAAGTGTGACCTGGCTGCACGCACTGTGGAATCTCGGCAGGCTCAGGA CCTCGCCCGAAGCTACGGCATCCCCTACATCGAGACCTCGGCCAAGACCCG GCAGGGAGTGGAGGATGCCTTCTACACGTTG

Table 2.5. qRT-PCR reagents for *KRAS* assay

Assay component	Sequence
Forward primer	GACTCTGAAGATGTACCTATGGTCCTAGTA
Reverse primer	TCATCAACACCCTGTCTTGTCTTT
Probe	AGACACAAAACAGGCTCAGGACTTAGCAAGAA
Amplicon	GACTCTGAAGATGTACCTATGGTCCTAGTAGGAAATAAATGTGATTTGCCTTCT AGAACAGTAGACACAAAACAGGCTCAGGACTTAGCAAGAAGTTATGGAATTCC TTTTATTGAAACATCAGCAAAGACAAGACAGGGTGTTGATGA

Table 2.6. qRT-PCR reagents for *NRAS* assay

Assay component	Sequence
Forward primer	GATGTACCTATGGTGCTAGTGGGA
Reverse primer	TTCTCTTACCAGTGTGTAAAAAGCATCT
Probe	TACAAAACAAGCCCACGAACTGGCCAAG
Amplicon	GATGTACCTATGGTGCTAGTGGGAAACAAGTGTGATTTGCCAACAAGGACAGT TGATACAAAACAAGCCCACGAACTGGCCAAGAGTTACGGGATTCCATTCATTG AAACCTCAGCCAAGACCAGACAGGGTGTTGAAGATGCTTTTTACACACTGGTA AGAGAA

CHAPTER 3.

STIMULATION OF EPHA2 WITH EPHRIN-A1 SUPPRESSES MAPK AND PI3K SIGNALING

3.1. INTRODUCTION

3.1.i. Eph receptors and ephrin ligands

Eph receptors comprise the largest family of receptor tyrosine kinases (RTKs) in the human genome, with 14 Eph receptors and 8 membrane associated ephrin (Eph receptor-interacting) ligands currently described. Eph receptors are divided into two classes based on sequence similarity and ligand binding specificity. In general, EphA receptors interact promiscuously with glycosylphosphatidylinositol (GPI)-tethered ephrin-A ligands, whereas EphB receptors interact promiscuously with transmembranous ephrin-B ligands. Exceptions include the EphA4 and EphB2 receptors, which can also interact with ephrin-Bs and ephrin-A5, respectively, as well as the EphB4 receptor, which only interacts with ephrin-B2 (Table 3.1).

The extracellular portion of Eph receptors is comprised of a globular amino-terminal ligand-binding domain (LBD) followed by a cysteine-rich domain (CRD) and two fibronectin type III repeats (FN3). The intracellular portion of Eph receptors is comprised of a regulatory juxtamembrane region followed by a conserved tyrosine kinase domain, a sterile α motif (SAM) protein-protein interaction domain and a C-terminal PDZ binding motif (Himanen et al., 2009; Himanen et al., 2010). Ephrin ligands are comprised of a 20 kDa extracellular receptor-binding domain; B-class ephrins also contain a short intracellular cytoplasmic region (Figure 3.1).

Table 3.1. Interactions between Eph receptors and ephrin ligands

Eph Receptor	Interacting ephrin ligands
EphA1, EphA2, EphA3, EphA5, EphA6, EphA7, EphA8, EphA10	ephrin-A1, ephrin-A2, ephrin-A3, ephrin-A4, ephrin-A5
EphA4	ephrin-A1, ephrin-A2, ephrin-A3, ephrin-A4, ephrin-A5, ephrin-B1, ephrin-B2, ephrin-B3
EphB1, EphB3, EphB6	ephrin-B1, ephrin-B2, ephrin-B3
EphB2	ephrin-B1, ephrin-B2, ephrin-B3, ephrin-A5
EphB4	ephrin-B2

The interaction between an Eph receptor and its membrane-associated ephrin ligand occurs between two apposed cells at sites of cell-cell contact, and propagates bidirectional signals. "Forward signaling" depends on the kinase activity of the Eph receptor and results in signaling changes in the receptor-expressing cell. Eph receptors can also propagate kinase-independent and cell non-autonomous "reverse signaling" events through their respective ephrin ligands, resulting in signaling changes in the ligand-expressing cell (Pasquale, 2010; Wykosky and Debinski, 2008). Through these interactions, Eph receptors and ligands mediate cellular adhesion, repulsion, motility, and migration in a variety of biological settings, including tissue patterning of the nervous, skeletal and vascular systems.

Eph-mediated signaling is a multi-step process that commences when a catalytically repressed Eph receptor binds its ephrin ligand in *trans* to form a high-affinity heterodimer. Heterodimer pairs tetramerize to form 2:2 heterotetramers, and at high concentrations oligomerize to form higher order signaling clusters (Himanen et al., 2007). Ligand binding and subsequent receptor clustering promote *trans* phosphorylation of the cytoplasmic portion of Eph receptors (Himanen et al., 2007). Phosphorylation of tyrosine kinase residues in the juxtamembrane region relieves autoinhibition of the kinase domain, allowing for stimulation of kinase activity. As with other receptor tyrosine kinases, ligand stimulation and subsequent Eph receptor tyrosine kinase activation results in the recruitment of signaling and adaptor proteins to the phosphorylated receptor.

Eph receptor activation and signaling can be studied *in vitro* through the use of soluble recombinant ephrin ligand. Because effective activation of Eph receptor kinase activity requires multimerization, the recombinant ephrin ligand is dimerized by fusion to the Fc portion of IgG (ephrin-Fc), and can be pre-clustered via incubation with anti-Fc antibodies in order to more closely recapitulate the conditions *in vivo* (Wykosky and Debinski, 2008).

Several reports have suggested that A-class receptor/ligand pairs interact with a higher affinity than their B-class counterparts (Himanen et al., 2004; Himanen et al., 2009; Pabbisetty et al.,

2007). This can be explained in part by the difference in binding modes between A-class and B-class receptor/ligand pairs. B-class receptor/ligand recognition proceeds through an "induced-fit" mechanism, whereby the ligand binding domain of the EphB receptor rearranges upon ligand binding, requiring energy to generate an interaction surface complementary to the receptor-binding domain of the ephrin-B ligand. On the other hand, A-class receptor/ligand recognition proceeds through a "lock, key and latch" mechanism, in which the receptor and ligand are already complementary to each other in shape and chemical nature and do not require any conformational changes for interaction (Himanen et al., 2009). These models suggest that A-class receptors can be activated by their ligands more readily than B-class receptors. Indeed, activation of EphB2 *in vitro* requires pre-clustering of recombinant ephrin-B1-Fc prior to receptor stimulation. In contrast, EphA2 can be fully activated by dimeric ephrin-A1-Fc, and furthermore, pre-clustering does not promote a significant increase in receptor activation (Himanen et al., 2009). Additionally, recent evidence suggests that soluble, monomeric ephrin-A1 is sufficient to activate EphA2 kinase activity (Wykosky et al., 2008).

3.1.ii. The EphA2 receptor tyrosine kinase

EphA2 is a 130 kDa protein comprised of 976 amino acids, and was identified by Lindberg and Hunter in a screen for epithelial cell tyrosine kinases (Lindberg and Hunter, 1990). *EPHA2* is located within the chromosomal region 1p36.13 and is expressed in tissues with a high proportion of dividing epithelial cells, including skin, lung, and small intestines (Ruiz and Robertson, 1994; Sulman et al., 1997). In fact, the receptor was initially named epithelial cell kinase (ECK) due to its wide distribution on adult epithelial cells *in vitro* and *in vivo*.

EphA2 knockout mice are viable and fertile and show no overt anomalies, suggesting other Eph receptors may be able to compensate for EphA2 during development (Chen et al., 1996; Mitchell et al., 2001). However, one line of *EphA2* knockout mice displayed short, kinked tails, implicating the receptor in notochord development (Naruse-Nakajima et al., 2001). Additionally, two independent strains of *EphA2* knockout mice develop progressive cortical cataract, suggesting

EphA2 is important in maintaining lens clarity with age (Jun et al., 2009). Indeed, mutations in *EPHA2* have been identified in patients with both age-related and autosomal recessive congenital cataracts (Hattersley et al., 2010; Jun et al., 2009; Kaul et al., 2010; Shiels et al., 2008; Zhang et al., 2009).

EphA2 interacts promiscuously with all five ephrin-A ligands; however, *in vitro* studies widely utilize recombinant ephrin-A1-Fc to examine signaling downstream of the receptor. Stimulation of EphA2 by ephrin-A1 (or by recombinant ephrin-A1-Fc) induces receptor activation and subsequent internalization and degradation. Timecourse studies indicate that activated EphA2 is internalized within 20 minutes (Walker-Daniels et al., 2002). Additionally, EphA2 protein levels begin to decline 30 minutes after activation, and are negligible 4 to 24 hours after activation, although later timepoints have not been examined (Wykosky et al., 2008).

Signaling downstream of EphA2 activation is dependent on both cell type and cellular context. For example, activation of EphA2 in endothelial cells and epithelial cells results in diametrically opposite effects on MAPK and PI3K signaling, cell proliferation and motility (Brantley-Sieders et al., 2004; Macrae et al., 2005; Menges and McCance, 2008; Miao et al., 2000; Miao et al., 2009; Pandey et al., 1994; Pasquale, 2010). Attributes such as these make EphA2 a unique and challenging receptor to study.

3.1.iii. Regulation of EphA2 expression in cancer

EphA2 is highly expressed in several tumor types including glioblastoma, melanoma, colon, prostate, lung and breast cancers, and its expression correlates with increased metastasis and poor prognosis (Kamat et al., 2009; Kinch and Carles-Kinch, 2003; Pasquale, 2010; Thaker et al., 2004; Walker-Daniels et al., 1999; Wykosky and Debinski, 2008). Although EphA2 is highly expressed in many cancers, there has been little evidence of *EPHA2* gene amplification. Additionally, few cancer-associated mutations in *EPHA2* have been reported to date. The Catalog of Somatic Mutations in Cancer (COSMIC; www.sanger.ac.uk/genetics/CGP/cosmic/)

database includes only six samples with mutations in *EPHA2*, and the functional consequence of these particular mutations have yet to be characterized (Table 3.2). A recent study characterized the G391R mutation in the receptor, which was detected in two squamous cell carcinomas and one non-small cell lung cancer cell line. EphA2 G391R confers increased signaling through Src and mTOR as well as increased survival and invasiveness (Faoro et al., 2010). The G391R mutation is located in the fibronectin type III repeat of EphA2, and the mechanism by which the mutation alters protein function is not fully understood.

Table 3.2. Cancer associated mutations in *EPHA2*

Sample Name	Tissue Type	Amino acid mutation
PD1482a	Stomach	G777S
TCGA-24-1103	Ovary	W912C
TCGA-24-1419	Ovary	R957C
Br27P	Brain	P817L
TCGA-02-0114	Brain	G111D
16835	Lung	E663*

High levels of EphA2 expression on tumor cells may result from increased transcription of *EPHA2*. Several studies have shown that signaling pathways commonly hyperactivated in cancer induce transcription of *EPHA2*. For example, *EPHA2* is a direct transcriptional target of the Ras/MAPK pathway, and EphA2 levels are increased in Ras-transformed cells and in transgenic mice that overexpress Ras (Andres et al., 1994; Macrae et al., 2005; Pratt and Kinch, 2003). Similarly, high levels of EGFR signaling induce EphA2 gene and protein expression (Larsen et al., 2007; Larsen et al., 2010; Ramnarain et al., 2006). *EPHA2* is also regulated by the homeobox transcription factors HOXA1 and HOXB1, which have been reported to be up-regulated in cancer (Chen and Ruley, 1998). Finally, an analysis of the *EPHA2* promoter revealed DNA damage-responsive p53-binding sites, implicating p53 in the regulation of EphA2 expression (Dohn et al., 2001; Jin et al., 2006). In contrast, both c-Myc and estrogen receptor signaling – which are often lost in the most aggressive cancers – have been shown to repress *EPHA2* transcription (Zelinski et al., 2002).

High levels of EphA2 expression on tumor cells may also result from increased protein stability. It has been reported that overexpression of the phosphatase LMW-PTP decreases the tyrosine phosphorylation content of EphA2, thereby preventing internalization and contributing to the accumulation of the receptor at the cell surface (Kikawa et al., 2002). Alternatively, the inaccessibility of ephrin ligands to the EphA2 receptor may also contribute to receptor accumulation. The lack of adequate cell-cell contacts in aggressive cancer cells might prevent juxtacrine signaling between apposing Eph receptor and ephrin ligand expressing cells and may therefore stabilize receptor expression. Along these lines, it has been reported that the cell adhesion molecule E-cadherin promotes the interaction between EphA2 and ephrin-A1 at epithelial junctions, perhaps due to its ability to promote proper cell-cell contacts (Orsulic and Kemler, 2000; Zantek et al., 1999).

Cancer cells that express high levels of EphA2 oftentimes do not express ephrin ligand, thereby precluding any potential juxtacrine signaling within a tumor mass. For example, expression of the EphA2 receptor and its ligand ephrin-A1 is inversely proportional in a panel of 28 human breast cancer cell lines (Macrae et al., 2005). Eight of twenty-eight cell lines highly express the EphA2 receptor, but do not express the ephrin-A1 ligand. Conversely, the remaining cell lines express the ephrin-A1 ligand, but do not express the EphA2 receptor. A similar reciprocal pattern of EphA2 receptor and ephrin ligand expression is observed in glioblastoma multiforme cancer cell lines and patient samples (Wykosky et al., 2005). Therefore, in each of these cell types, EphA2 signaling is disrupted, either by loss of receptor or loss of ligand expression.

The reciprocal expression pattern of receptor and ligand observed in these cell lines may be due to a feedback mechanism in which receptor expression suppresses ligand expression, and vice versa. siRNA-mediated depletion of ephrin-A1 in ligand-expressing breast cancer cell lines profoundly up-regulates EphA2 protein expression (Macrae et al., 2005). Conversely, ectopic ephrin-A1 expression in receptor-expressing breast cancer and glioblastoma multiforme cell lines suppresses endogenous EphA2 protein levels (Macrae et al., 2005; Wykosky et al., 2005). The exact mechanism by which this feedback occurs has yet to be fully elucidated.

3.1.iv. Ligand-dependent tumor suppressive properties of EphA2

The functional consequence of EphA2 overexpression in cancer has been widely investigated over the past several years. Early studies revealed distinct differences between EphA2 expressed on untransformed and transformed cells. In untransformed epithelial cells, EphA2 is expressed at low levels, is tyrosine phosphorylated, and localized to points of cell-cell contact (Zantek et al., 1999). In transformed cells, EphA2 is highly expressed but is not activated, as judged by the low level of tyrosine phosphorylation in the kinase domain of the receptor. Many of the scenarios described previously – an abundance of tyrosine phosphatases, the inability of juxtacrine signaling to persist due to a lack of proper cell-cell contacts, and a loss of ephrin ligand expression – may account for why EphA2 remains unphosphorylated and inactivated on cancer cells.

Most receptor tyrosine kinases overexpressed or amplified in cancer contribute to the tumorigenic phenotype through constitutive activation and increased downstream signaling. EphA2 is unique amongst receptor tyrosine kinases in that its abundant overexpression on cancer cells is accompanied by a disruption in signaling and a lack of tyrosine kinase activation. One explanation for this phenomenon is that EphA2 is reported to have ligand-dependent tumor suppressive properties – when activated by its ligand ephrin-A1, the EphA2 receptor modulates downstream signaling pathways to suppress cell proliferation, motility and invasion. Therefore, cancer cells that have disrupted EphA2 signaling by any of the aforementioned mechanisms are able to evade the tumor suppressive properties of the activated receptor.

Importantly, EphA2 expressed on cancer cells still retains the potential to become activated by ephrin ligand. It has been well documented that upon stimulation of EphA2-expressing cancer cells with recombinant ephrin-A1-Fc, EphA2 is activated and downstream signaling events are initiated, followed by receptor internalization and degradation (Carter et al., 2002; Duxbury et al., 2004b; Shao et al., 1995; Walker-Daniels et al., 2002; Wykosky et al., 2005). Activation of EphA2

with ephrin-A1 suppresses many tumorigenic properties of cancer cells, and results in a reduction in cell proliferation, motility and invasiveness *in vitro*, impaired three-dimensional growth in agar and reconstituted basement membrane matrix, as well as decreased tumor growth *in vivo* (Duxbury et al., 2004b; Miao et al., 2000; Miao et al., 2009; Miao et al., 2003; Miao et al., 2005; Miao et al., 2001; Noblitt et al., 2004; Wykosky et al., 2005; Zelinski et al., 2001). Mechanistically, stimulation with ephrin-A1 could elicit these tumor suppressive properties of EphA2 by causing internalization and degradation of the receptor and/or by inducing anti-oncogenic signaling events downstream of receptor activation. Along these lines, EphA2 is known to down-regulate several oncogenic signaling pathways upon ligand stimulation, including the Ras/MAPK, PI3K, integrin, and Rac signaling pathways (Miao et al., 2000; Miao et al., 2009; Miao et al., 2003; Miao et al., 2005; Miao et al., 2001).

Genetic evidence also supports a tumors suppressive role for EphA2. *EphA2* knockout mice are significantly more susceptible to chemically-induced skin carcinogenesis, suggesting a tumor suppressive role for the receptor (Guo et al., 2006). Additionally, the chromosomal region within which EphA2 resides is often deleted in cancers of neuroectodermal origin, further supporting this notion (Miozzo et al., 2000; Riva et al., 2003; Sulman et al., 1997).

3.1.v. Ligand-independent oncogenic properties of EphA2

Despite the ligand-dependent anti-tumorigenic properties of EphA2, several studies suggest a ligand-independent oncogenic role for the receptor as well. An early study by Zelinski *et al.* reported that ectopic overexpression of EphA2 was sufficient to transform the MCF-10A mammary epithelial cell line (Zelinski et al., 2001). Additionally, depletion of EphA2 by siRNA reduced cellular invasiveness *in vitro* and suppressed tumor growth *in vivo* (Carles-Kinch et al., 2002; Duxbury et al., 2004a; Duxbury et al., 2004b; Miao et al., 2009) suggesting that overexpression of the receptor may contribute to the tumorigenic phenotype.

A recent study by Miao *et al.* demonstrates how EphA2 can both promote and inhibit cancer cell migration and invasion (Miao et al., 2009). Cell migration was significantly enhanced by ectopic overexpression of EphA2, and conversely was suppressed by shRNA-mediated depletion of EphA2 in several cell lines. The authors found that EphA2 is a substrate of the serine/threonine kinase Akt, and that phosphorylation of EphA2 at serine 897 by Akt is required for the ligand-independent promotion of cell migration by the receptor. Furthermore, kinase activity of the EphA2 receptor is not required for this effect. Interestingly, kinase activation of EphA2 with ephrin-A1-Fc reduces Akt activity, abolishes the phosphorylation of EphA2 at serine 897, and correlates with a reduction in chemotaxis. Therefore, cell migration and invasion is promoted by EphA2 in a ligand-independent manner, and inhibited by EphA2 in a ligand-dependent manner. This study elegantly illustrates how ligand stimulation and subsequent kinase activation transform EphA2 from an oncogene to a tumor suppressor.

3.1.vi. Therapeutic targeting of EphA2 in cancer

The EphA2 receptor tyrosine kinase is highly expressed on the surface of many different human cancer cells, making it an attractive candidate for targeted therapeutics. EphA2 is unique amongst receptor tyrosine kinases in that it exhibits both ligand-independent tumor-promoting properties and ligand-dependent tumor-suppressive properties. Various strategies have been employed to exploit these properties for therapeutic purposes.

One approach to subvert the tumor-promoting properties of EphA2 involves silencing the expression of the receptor by RNA interference. Several studies demonstrate that silencing *EPHA2* reduces cancer cell growth, motility and invasion *in vitro* and *in vivo* (Duxbury et al., 2004a; Landen et al., 2005a; Landen et al., 2005b; Landen et al., 2006b). As with other RNAi-based therapies, the promise of this approach will depend on the efficient delivery of siRNA to EphA2-expressing cancer cells.

A second strategy to exploit EphA2 for therapeutic purposes involves promoting the tumor-suppressive properties of the receptor by stimulating tyrosine kinase activity. Therapeutic approaches to induce EphA2 kinase activation include the use of agonistic antibodies as well as adenoviral delivery of ephrin-A1 to EphA2-expressing cancer cells (Carles-Kinch et al., 2002; Landen et al., 2006a; Noblitt et al., 2004; Noblitt et al., 2005). Activation of EphA2 by these therapeutic approaches could in theory suppress cancer cell tumorigenicity by inducing anti-oncogenic signaling events downstream of receptor activation as well as by inducing receptor internalization and degradation. However, the utility of this approach is complicated by the fact that EphA2 has diametrically opposing functional roles in different cell types. For instance, activation of EphA2 on endothelial cells is known to activate both MAPK and PI3K signaling and promote angiogenesis (Brantley-Sieders et al., 2004; Pandey et al., 1994). Therapies designed to stimulate EphA2 kinase activity would therefore require selective delivery to epithelial tumor cells.

A third strategy involves the selective delivery of cytotoxic compounds to EphA2-expressing cancer cells. To date, there have been two therapeutic agents developed that utilize this approach. The first example is ephrin-A1-PE38QQR, in which the ligand ephrin-A1 is conjugated to a mutated form of *Pseudomonas aeruginosa* exotoxin A (Wykosky et al., 2007). Though the conjugate was successful at reducing the proliferation of EphA2-expressing glioblastoma, breast and prostate cancer cells *in vitro*, one caveat of this approach is that it is not absolutely specific for EphA2, as all A-class Eph receptors bind promiscuously to ephrin-A1. A second therapeutic agent that addresses this concern is an antibody-drug conjugate comprised of an EphA2 agonistic monoclonal antibody conjugated to a microtubule inhibitor. The compound, 1C1-maleimidocaproyl-monomethylauristatin (1C1-mcMMAF), specifically induces tyrosine phosphorylation, internalization, and degradation of the EphA2 receptor. 1C1-mcMMAF selectively attenuated the growth of EphA2-expressing tumor xenografts *in vivo* and is currently under further clinical investigation.

3.1.vii. Suppression of MAPK and PI3K signaling by stimulation of the EphA2 receptor tyrosine kinase

The MAPK and PI3K pathways are critically important in regulating cellular processes such as growth, proliferation, and survival, and are commonly deregulated in cancer. Our laboratory identified the receptor tyrosine kinase EphA2 as a direct transcriptional target of the MAPK pathway, and demonstrated that stimulation of EphA2 with ephrin-A1-Fc attenuates MAPK signaling at the level of ERK phosphorylation. These results indicate that EphA2 maintains a negative feedback loop to regulate MAPK activity in a ligand-dependent manner: high MAPK activity stimulates EphA2 expression, and stimulation of EphA2 by its ligand ephrin-A1 then inhibits MAPK signaling. We have also found that stimulation of EphA2 with ephrin-A1-Fc attenuates PI3K signaling at the level of Akt phosphorylation. These findings have potential therapeutic implications: stimulating the EphA2 receptor with its ligand ephrin-A1 may present a viable method to suppress the tumorigenicity of cancer cells that overexpress EphA2 and are dependent on high MAPK and PI3K signaling. Therefore, the aim of this work is to determine the molecular mechanism(s) by which ligand stimulated EphA2 interferes with MAPK and PI3K signaling. Understanding these mechanisms is critically important in determining which tumor cells might be most sensitive to a therapeutic approach based on the stimulation of EphA2 tyrosine kinase activity.

EphA2 has been shown to be both necessary and sufficient in mediating MAPK pathway inhibition downstream of ephrin-A1-Fc stimulation (Guo et al., 2006). Stimulation with ephrin-A1-Fc reduces ERK phosphorylation in MEFs isolated from wild type and *EphA2* heterozygous mice, but does not affect ERK phosphorylation in MEFs isolated from *EphA2* knockout mice, suggesting EphA2 is necessary to mediate this effect. Ectopic expression of EphA2 in MEFs derived from *EphA2* knockout mice rescues the ability of ephrin-A1-Fc to reduce ERK phosphorylation, demonstrating that EphA2 is sufficient to mediate MAPK pathway inhibition downstream of ligand stimulation.

The requirement for EphA2 in mediating PI3K pathway inhibition appears to be cell line dependent, as conflicting results have been reported in the literature. EphA2 appears to be necessary for attenuating PI3K signaling downstream of ligand stimulation in the PC3 prostate cancer cell line, as siRNA-mediated depletion of EphA2 abolishes the ability of ephrin-A1-Fc stimulation to reduce Akt phosphorylation (Yang et al., 2011). It should be noted that in this cell line, siRNA-mediated depletion of EphA2 does not abolish the ability of ephrin-A1-Fc stimulation to reduce ERK phosphorylation, suggesting that the activation of other EphA kinases may also mediate MAPK pathway inhibition in PC3 cells. Studies utilizing wild-type and *EphA2* null mouse keteratinocytes demonstrate a requirement for EphA2 in attenuating ERK phosphorylation downstream of ephrin-A1-Fc stimulation. However, in both the wild-type and *EphA2* null keratinocytes, Akt phosphorylation levels were not affected by ephrin-A1-Fc stimulation, suggesting that ligand stimulated EphA2 acts specifically on the MAPK pathway in this cell type (Guo et al., 2006).

Ephrin-A1-Fc interacts promiscuously with all A-class Eph receptors. However, treatment of EphA2-expressing cells with selective agonists of EphA2, including the YSA peptide and the monoclonal antibody 1C1, attenuates both ERK and Akt phosphorylation in a dose-dependent manner in PC3 prostate cancer cells, demonstrating that the specific stimulation of EphA2 receptor tyrosine kinase activity does indeed lead to the attenuation of MAPK and PI3K signaling. (Yang et al., 2011).

The molecular mechanism(s) by which ligand stimulated EphA2 suppresses ERK and Akt phosphorylation is currently unknown. The small GTPase Ras is a common upstream regulator of the MAPK and PI3K signaling pathways, and represents a possible point of convergence downstream of EphA2 activation and upstream of ERK and Akt phosphorylation. Indeed, earlier studies demonstrate that stimulation of EphA kinases with ephrin-A1-Fc results in a reduction in Ras-GTP levels and a concomitant decrease in MEK and ERK phosphorylation (Miao et al., 2001). Additionally, stimulation of EphA kinases with ephrin-A1-Fc attenuates the activation of MAPK signaling by EGF, PDGF, and VEGF, and inhibits cell proliferation. Furthermore, ectopic

overexpression of constitutively active K-Ras blocks the ability of ligand stimulated EphA2 to reduce ERK phosphorylation levels or inhibit cell growth. Of note, the authors did not examine any markers of PI3K pathway activity in this study.

Activation of another Eph family receptor tyrosine kinase, EphB2, also inhibits the MAPK pathway at the level of Ras. The ability of ligand stimulated EphB2 to suppress Ras signaling is dependent on the autophosphorylation of two conserved tyrosine residues in the juxtamembrane region of the receptor as well as subsequent kinase activation (Elowe et al., 2001). Following stimulation with ephrin-B1-Fc, the GTPase activating protein p120 RasGAP binds phosphorylated tyrosine residues within the juxtamembrane region of the activated EphB2 receptor through its two SH2 domains (Elowe et al., 2001; Holland et al., 1997). Additionally, p120 RasGAP also binds the activated EphB2 receptor indirectly through the adaptor protein $p62^{dok}$. A truncated form of p120 RasGAP, which lacks the C-terminal catalytic GAP domain but retains the N-terminal SH2 and SH3 domains, was used as a dominant negative construct to show that the catalytic activity of p120 RasGAP is required for ligand stimulated EphB2 to suppress MAPK signaling (Elowe et al., 2001). Taken together, these data support a model in which p120 RasGAP is recruited to the activated EphB2 receptor following ephrin-B1-Fc stimulation and promotes the hydrolysis of bound GTP on Ras, thereby attenuating MAPK pathway activity.

Whether p120 RasGAP also signals downstream other Eph receptors to suppress MAPK signaling has yet to be determined. The SH2 domains of p120 RasGAP have been shown to interact with a phosphorylated tyrosine residue within the consensus sequence (phospho)YXXPXD. Proteins that have a well established interaction with p120 RasGAP contain an aspartic acid at the +5 position relative to the phosphorylated tyrosine, and it has been argued that an acidic amino acid at this position determines binding affinity for p120 RasGAP (Hock et al., 1998). The EphB2 receptor, which has been demonstrated to bind p120 RasGAP, has a conservative substitution of glutamic acid at this position. In contrast, the EphA4 receptor does not bind p120 RasGAP, and contains a non-conservative substitution of glutamine at the +5 position (Ellis et al., 1996). Based on its amino acid sequence, EphA2 is not predicted to directly

bind p120 RasGAP either, as it also contains the non-conservative substitution of glutamine at this position (Hock et al., 1998).

Despite this, several studies have implicated p120 RasGAP as an important signaling protein downstream of EphA2 receptor activation. Studies utilizing MEFs isolated from wild-type and p120 RasGAP knockout mice suggest that p120 RasGAP is required for the suppression of MAPK signaling by endogenous EphA receptors (Tong et al., 2003). Stimulation of wild-type MEFs with ephrin-A1-Fc leads to a reduction in ERK phosphorylation. However, stimulation of p120 RasGAP null MEFs with ephrin-A1-Fc has no affect on ERK phosphorylation levels. It has not been determined whether reconstitution of the knockout MEFs with ectopic p120 RasGAP is sufficient to rescue the ability of ligand stimulated EphA receptors to suppress MAPK signaling. Another study performed in PC3 prostate cancer cells reports that siRNA-mediated depletion of p120 RasGAP abrogates the ability of ephrin-A1-Fc stimulation to attenuate the levels of EGF-induced ERK phosphorylation (Parri et al., 2005). The authors of this study also report an interaction between p120 RasGAP and activated EphA2 that is dependent on ephrin-A1-Fc stimulation and phosphorylation of tyrosine 594 located within the juxtamembrane region of the EphA2 receptor.

PI3K can be activated by upstream receptor tyrosine kinases or through an interaction with activated Ras. Therefore, it is plausible that ligand stimulation of EphA2 attenuates PI3K signaling indirectly by down-regulating Ras activity. The levels of Akt phosphorylation, or other markers of PI3K pathway activity, were not assessed in the aforementioned studies, and so it remains to be determined whether the suppression of Ras signaling by ligand stimulated EphA2 is responsible for the decreases observed in Akt phosphorylation.

Alternatively, MAPK and PI3K signaling may be differentially regulated by ligand stimulated EphA2. A recent study by Yang *et al.* argues that ligand stimulated EphA2 regulates Akt signaling independently of Ras. The authors present two lines of evidence to support this hypothesis. First, activation of EphA2 suppresses Akt but not ERK phosphorylation in the MDA-

MB-231 breast cancer cell line, which harbors activating mutations in both *KRAS* and *BRAF*. Secondly, ectopic overexpression of constitutively active H-Ras in the PC3 prostate cancer cell line abrogates the ability of activated EphA2 to suppress ERK, but not Akt, phosphorylation. In addition, the authors show that activated EphA2 can suppress Akt phosphorylation downstream of endogenous and ectopic constitutively active PI3K, and that the phosphatases PTEN, SHIP2, PHLPP1 and PHLPP2 are not required to mediate the effect. The results of experiments utilizing various phosphatase inhibitors are consistent with the involvement of a novel PP1-like phosphatase in mediating the attenuation of Akt phosphorylation downstream of EphA2 activation (Yang et al., 2011). Although the exact phosphatase has not yet been identified, this study provides strong evidence that the MAPK and PI3K signaling pathways are independently regulated by ligand activated EphA2.

Our laboratory had made similar observations prior to the publication of this study. In particular, we also observed that ligand stimulation of EphA2 reduced Akt but not ERK phosphorylation in the MDA-MB-231 breast cancer cell line. In addition, we observed an interesting phenotypic effect on MDA-MB-231 cells grown in the presence of ephrin-A1-Fc. While untransformed mammary epithelial cells form organized spherical structures when cultured in a three dimensional reconstituted basement membrane matrix, the MDA-MB-231 cell line forms highly invasive, disorganized outgrowths (Han et al., 2010; Kenny et al., 2007). However, we have shown that when grown in the presence of ephrin-A1-Fc, the MDA-MB-231 cell line forms more organized, spherical structures that resemble normal, untransformed mammary epithelial cells (R. Neve, M. Macrae, J. Gray; data not shown). We were intrigued to make this observation in a cancer cell line with activating mutations in both *KRAS* and *BRAF*, as it suggests that stimulation of EphA2 with ephrin-A1 could potentially inhibit the tumorigenicity of cancer cells with deregulated MAPK or PI3K signaling.

At the onset of this research project, many of the research discoveries described above had not yet been published. The goal of this work was two-fold:

a) To determine the molecular mechanism(s) by which ligand stimulated EphA2 suppresses MAPK and PI3K signaling

b) To determine whether ligand stimulation of EphA2 affects Akt and ERK phosphorylation in cancer cell lines with endogenous MAPK and PI3K pathway mutations

These studies will help determine which tumor cells might be most sensitive to a therapeutic approach based on the stimulation of EphA2 tyrosine kinase activity, and in addition will enhance our understanding of the crosstalk amongst the EphA2, Ras, PI3K and MAPK signaling pathways.

3.2. RESULTS

3.2.i. Stimulation with ephrin-A1-Fc suppresses Ras, MAPK and PI3K signaling

It has previously been reported that stimulation of EphA kinases with ephrin-A1-Fc attenuates the activation of MAPK signaling by various growth factors, including EGF, PDGF, and VEGF (Miao et al., 2001). The breast cancer cell line HBL100 was utilized to confirm and extend upon these observations. The HBL100 cell line expresses high levels of the EphA2 receptor, expresses little to no ephrin-A1 ligand (Macrae et al., 2005), and importantly, has no known mutations within the MAPK or PI3K pathways. Stimulation with ephrin-A1-Fc suppresses the basal levels of MAPK and PI3K signaling in serum starved HBL100 cells. Furthermore, EGF-, insulin- and PDGF-induced activation of MAPK and PI3K signaling is also attenuated by stimulation with ephrin-A1-Fc (Figure 3.2 A). These results suggest that ligand stimulated EphA2 interferes with MAPK and PI3K signaling downstream of growth factor receptor activation.

The small GTPase Ras is a common upstream regulator of the MAPK and PI3K signaling pathways, and represents a possible point of convergence downstream of Eph receptor activation and upstream of ERK and Akt phosphorylation. Previous studies report that stimulation of EphA kinases with ephrin-A1-Fc results in a reduction of Ras-GTP levels and a concomitant decrease in MEK and ERK phosphorylation (Miao et al., 2001). Consistent with these findings, stimulation of HBL100 cells with ephrin-A1-Fc results in a reduction of both basal and EGF-induced Ras-GTP levels and correlates with a decrease in ERK and Akt phosphorylation (Figure 3.2 B). Therefore, one potential mechanism by which ephrin-A1-Fc suppresses MAPK and/or PI3K signaling is through the suppression of the upstream regulator Ras.

In addition, stimulation with ephrin-A1-Fc inhibits PI3K signaling, but not MAPK signaling, in cell lines with activating *BRAF* mutations (Figures 3.2 C, 3.3 A). These results suggest that ephrin-A1-Fc interferes with MAPK signaling upstream of BRAF and are consistent with the finding that ephrin-A1-Fc suppresses Ras activity (Figure 3.2 D).

3.3.ii. Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling in cancer cell lines harboring oncogenic RAS mutations

Oncogenic Ras is constitutively locked in a GTP-bound state and is insensitive to the action of GTPase activating proteins. Because ephrin stimulation reduces the levels of Ras-GTP, one would predict that signaling would be unaffected by ephrin stimulation in cancer cells endogenously expressing oncogenic Ras. However, stimulation with ephrin-A1-Fc suppresses both MAPK and PI3K signaling in cancer cell lines harboring oncogenic *RAS* mutations (Figure 3.3 A). This effect was observed regardless of cancer cell line origin or Ras isoform mutation. As expected, the oncogenic Ras isoform remains constitutively GTP-bound, regardless of serum starvation, EGF or ephrin-A1-Fc stimulation (Figure 3.3 A). Remarkably, the ephrin-A1-Fc-induced reduction of Akt and ERK phosphorylation correlates with a reduction in GTP-loading on the wild-type Ras isoforms. The effect of ephrin-A1-Fc stimulation in cell lines harboring

oncogenic *RAS* mutations is most prominent in the context of EGF stimulation, as basal levels of wild-type Ras-GTP are quite low in the serum starved state.

The results suggest that ephrin-A1-Fc can suppress signaling in cancer cell lines harboring oncogenic *RAS* mutations by modulating the activity of wild-type Ras. To conclusively demonstrate this, the *NRAS* mutant rhabdomyosarcoma cell line RD was transfected with siRNA to specifically deplete the expression of either wild-type or oncogenic Ras. siRNA-mediated depletion of the two wild-type Ras isoforms, H-Ras and K-Ras, prevents both EGF and ephrin-A1-Fc stimulation from modulating ERK signaling, demonstrating that the wild-type Ras isoforms are required to mediate this effect (Figure 3.3 B). In contrast, siRNA-mediated depletion of N-Ras markedly enhanced the ability of EGF and ephrin-A1-Fc to modulate ERK signaling, consistent with the notion that the fluctuations in downstream MAPK signaling are primarily mediated via the wild-type Ras isoforms in this cell line. Importantly, these results conclusively demonstrate that ephrin-A1-Fc stimulation suppresses MAPK signaling through the upstream GTPase Ras.

3.3.iii. Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling in cancer cell lines with oncogenic PI3K pathway mutations

Interestingly, siRNA-mediated depletion of the two wild-type Ras isoforms did not affect the ability of ephrin-A1-Fc stimulation to attenuate Akt phosphorylation (Figure 3.3 B). Although PI3K pathway activity can be modulated by Ras signaling, these results suggest that the activation of EphA kinases by ephrin-A1-Fc stimulation likely employs an additional independent mechanism to suppress PI3K signaling. These results are consistent with recently published data that implicate a serine/threonine phosphatase in the suppression of Akt signaling downstream of activated EphA2 (Yang et al., 2011)

To extend upon these observations, several cancer cell lines with PI3K pathway mutations were screened for sensitivity to ephrin-A1-Fc stimulation. Stimulation with ephrin-A1-Fc suppressed PI3K signaling downstream of ectopic and endogenous oncogenic PI3K harboring mutations in

either the helical or kinase domain of p110α (Figure 3.4 A and 3.4 B). These results suggest that ephrin signaling inhibits the PI3K pathway downstream of lipid production.

PTEN (phosphatase and tensin homologue) is a lipid phosphatase that counters the activity of PI3K by removing the 3' phosphate on PIP_3 to generate PIP_2. Results obtained from the PTEN-null cell lines BT549 and PC3 suggest that a functional copy of *PTEN* is not required to mediate the suppression of MAPK or PI3K signaling induced by ephrin-A1-Fc stimulation (Figures 3.4 C and 3.4 D). SHIP2 is another lipid phosphatase that removes the 5' phosphate from PIP_3 to generate $PI(3,4)P_2$. siRNA-mediated depletion of SHIP2 did not abrogate the ability of ephrin-A1-Fc to attenuate MAPK or PI3K signaling (data not shown). Taken together, these results suggest these two lipid phosphatases are not involved in mediating Akt dephosphorylation downstream of stimulation with ephrin-A1-Fc.

In summary, stimulation with ephrin-A1-Fc attenuates basal and growth factor-induced Akt phosphorylation at both Ser473 and Thr308 (Figure 3.2 A), and can do so in the presence of oncogenic PI3K and in the absence of lipid phosphatases (Figure 3.4). While the exact mechanism of PI3K pathway attenuation has not been elucidated, these results are consistent with data published recently implicating a novel serine/threonine phosphatase in the regulation of Akt signaling downstream of EphA2 activation (Yang et al., 2011).

3.2.iv. Neurofibromin is not required to mediate the suppression of MAPK or PI3K signaling induced by ephrin-A1-Fc stimulation

The mechanism by which stimulation with ephrin-A1-Fc suppresses Ras activity is unknown. One possible mechanism by which ephrin-A1-Fc might attenuate Ras signaling is through the recruitment of a GTPase activating protein (GAP). GAPs negatively regulate the levels of Ras-GTP by accelerating the hydrolysis of bound GTP to GDP. Neurofibromin is one of the more well-characterized mammalian GAPs. To test the requirement of neurofibromin downstream of ephrin signaling, ephrin stimulation experiments were performed in an *NF1*-deficient cell line and

in a cell line in which neurofibromin expression was depleted by siRNA (Figure 3.5). Stimulation with ephrin-A1-Fc suppresses both MAPK and PI3K signaling in the absence of neurofibromin, suggesting that neurofibromin is not required to mediate this effect.

3.2.v. Stimulation with ephrin-A1-Fc does not suppress MAPK or PI3K signaling in p120 RasGAP null MEFs

It has been previously shown that activation of another Eph receptor, EphB2, suppresses Ras signaling through the recruitment of p120 RasGAP. To test the requirement of p120 RasGAP downstream of ephrin-A1-Fc stimulation, wild-type and p120 RasGAP null MEFs were subjected to ephrin stimulation experiments. Consistent with observations previously reported (Tong et al., 2003), stimulation of wild-type MEFs with ephrin-A1-Fc attenuates ERK phosphorylation while stimulation of p120 RasGAP null MEFs with ephrin-A1-Fc has no effect on ERK phosphorylation (Figure 3.6 A). Importantly, reconstitution of the of p120 RasGAP null MEFs with ectopic p120 RasGAP is not sufficient to rescue the ability ephrin-A1-Fc to suppress MAPK or PI3K signaling (Figure 3.6 B). Therefore, the inability of ephrin-A1-Fc stimulation to suppress MAPK and PI3K signaling in the p120 RasGAP null MEFs is likely due to an issue in clonality rather than a direct result of a lack of p120 RasGAP expression. In further support of these observations, shRNA-mediated depletion of p120 RasGAP does not abrogate the ability of ephrin-A1-Fc to suppress MAPK or PI3K signaling in the wild-type MEFs (Figure 3.6 C).

3.2.vi. RNAi experiments do not conclusively support a role for p120 RasGAP in mediating the suppression of MAPK or PI3K signaling downstream of ephrin-A1-Fc stimulation

To extend these findings to human cancer cell lines, ephrin stimulation experiments were performed HBL100 cells depleted of p120 RasGAP using siRNA. Stimulation with ephrin-A1-Fc

suppresses both MAPK and PI3K signaling in HBL100 cells depleted of p120 RasGAP, suggesting p120 RasGAP is not required to mediate the effect (Figure 3.7 A).

It has been firmly established that p120 RasGAP functions downstream of activated EphB2 to suppress Ras signaling. Therefore, as a positive control, HBL100 cells stably expressing ectopic EphA2, EphB2 or kinase-dead EphB2 (EphB2 K653M) were subjected to ephrin stimulation experiments (Figure 3.7 B). HBL100 cells expressing EphB2 K653M did not respond to treatment with ephrin-B1-Fc, as predicted. In HBL100 cells expressing ectopic wild-type EphB2, stimulation with ephrin-B1-Fc reduced Akt and ERK phosphorylation. However, siRNA-mediated depletion of p120 RasGAP abrogated the ability of ephrin-B1-Fc to suppress MAPK and PI3K signaling in these cells, demonstrating the requirement for p120 RasGAP in this system. In contrast, siRNA-mediated depletion of p120 RasGAP did not consistently abrogate the ability of ephrin-A1-Fc to suppress MAPK or PI3K signaling in HBL100 cells expressing ectopic EphA2. These results suggest that p120 RasGAP likely does not participate in signaling downstream of activated EphA2.

3.3. DISCUSSION

In summary, stimulation with ephrin-A1-Fc suppresses the basal and growth factor-induced activation of Ras, MAPK and PI3K signaling. Importantly, the integrity of ephrin signaling is maintained in cancer cell lines harboring oncogenic mutations in *RAS* or within the PI3K pathway. As EphA2 is highly expressed in many cancers, these results hold promise that therapies designed to activate EphA2, by an agonist antibody or mimetic peptide for example, could potentially be a useful therapeutic strategy in tumors harboring these mutations.

The results presented here conclusively demonstrate that activation of EphA kinases suppress Ras activity. Consistent with these findings, the MAPK pathway is refractory to modulation by ephrin stimulation in cell lines harboring oncogenic mutations in *BRAF*. In spite of this, ephrin

signaling attenuates the growth factor-induced activation of MAPK and PI3K signaling in cancer cell lines with oncogenic *RAS* mutations, and does so by modulating the activity of the wild-type Ras isoforms. Experiments utilizing RNA interference to specifically deplete the expression of the wild-type Ras isoforms in cell lines harboring oncogenic *RAS* mutations demonstrate a requirement for wild-type Ras in mediating the suppression of MAPK – but not PI3K – signaling downstream of ephrin stimulation. These results conclusively show that ephrin stimulation suppresses MAPK signaling by attenuating the activity of the upstream GTPase Ras.

The mechanism by which ephrin stimulation inhibits Ras activity has yet to be determined. The experiments presented here suggest that p120 RasGAP and neurofibromin likely do not participate in signaling downstream of activated EphA2. Additionally, experiments utilizing RNA interference to deplete the expression of less characterized GTPase activating proteins have not uncovered a role for any of these proteins in mediating signaling downstream of ephrin stimulation (data not shown). As expected, siRNA-mediated depletion of p120 RasGAP in cells expressing ectopic EphB2 abrogated the ability of ephrin-B1-Fc to suppress both PI3K and MAPK signaling. These results suggest that the MAPK and PI3K signaling pathways are co-regulated by EphB2.

PI3K can be activated by upstream receptor tyrosine kinases or through a direct interaction with activated Ras. Therefore, it is plausible that ephrin-A signaling attenuates PI3K signaling indirectly by suppressing Ras activity. However, in the *NRAS* mutant cell line RD, siRNA-mediated depletion of the wild-type Ras isoforms abrogated the ability of ephrin-A1-Fc stimulation to affect ERK, but not Akt, phosphorylation. Therefore, the data presented here suggest that the MAPK and PI3K pathways are differentially regulated by ephrin-A signaling. Ephrin stimulation suppresses the basal and growth factor-induced phosphorylation of Akt at both Ser473 and Thr308, and does so in the presence of oncogenic PI3K and in the absence of regulatory lipid phosphatases. Although the exact molecular mechanism by which ephrin-A stimulation suppresses Akt phosphorylation has not been elucidated, recent studies implicate a novel PP1-like phosphatase in mediating the attenuation of Akt phosphorylation downstream of EphA2

activation (Yang et al., 2011). Importantly, this study is consistent with the findings presented here, and provides strong evidence that the MAPK and PI3K signaling pathways are independently regulated by ephrin-A signaling.

Perhaps one of the most striking findings of this study is the observation that the attenuation of wild-type Ras activity is sufficient to modulate downstream MAPK and PI3K signaling, despite the presence of an oncogenic *RAS* allele. It is a general belief in the field that MAPK and PI3K signaling is saturated by the constitutive activation of oncogenic Ras. However, the results presented here argue that this is not so, and furthermore, demonstrate that modulating the activation status of wild-type Ras is sufficient to result in fluctuations in downstream signaling.

Several studies have shown that activation of EphA2 with ephrin-A1 results in a reduction in cell proliferation, motility and invasiveness *in vitro*, impaired three-dimensional growth in agar and reconstituted basement membrane matrix, as well as decreased tumor growth *in vivo* (Duxbury et al., 2004b; Miao et al., 2000; Miao et al., 2009; Miao et al., 2003; Miao et al., 2005; Miao et al., 2001; Noblitt et al., 2004; Wykosky et al., 2005; Zelinski et al., 2001). However, an outstanding question is whether ephrin signaling might have similar consequences in cancer cells harboring oncogenic *RAS* mutations. Along these lines, a recent study demonstrated that EphA2 acts as a tumor suppressor in a mouse model of skin carcinogenesis driven by an activating mutation in *Hras* (Guo et al., 2006). Wild-type, *EphA2* heterozygous and *EphA2* knockout mice were subjected to the classic DMBA/TPA two step skin carcinogensis protocol, in which topical DMBA treatment leads to tumor initiation while application of TPA contributes to tumor promotion. Importantly, the tumors that develop in this model harbor an activating mutation in *Hras* at codon 61. With respect to wild-type mice, *EphA2* knockout mice developed skin tumors with increased frequency and shortened latency. Furthermore, tumor cells derived from *EphA2* knockout mice displayed an increased rate of cell proliferation and a more invasive phenotype, demonstrating that EphA2 can act as a tumor suppressor in the mouse skin, and can do so even in the context of an oncogenic Ras mutation. It is tempting to speculate that the tumor suppressive effects mediated by EphA2 in this system may have resulted from the suppression of wild-type N-Ras

and K-Ras signaling. Future experiments performed *in vitro* as well as in similar *in vivo* models will address some of these outstanding questions.

In conclusion, the discovery that wild-type Ras isoforms can contribute to the overall signaling output in cells harboring activating *RAS* mutations is a novel and surprising finding, and could lead to new strategies for treating cancers that contain oncogenic *RAS* mutations. Additionally, blocking the activation of wild-type Ras – by an agonist antibody targeted to EphA2, or by an ephrin mimetic peptide, for example – might be beneficial in tumors with high MAPK and PI3K pathway activity.

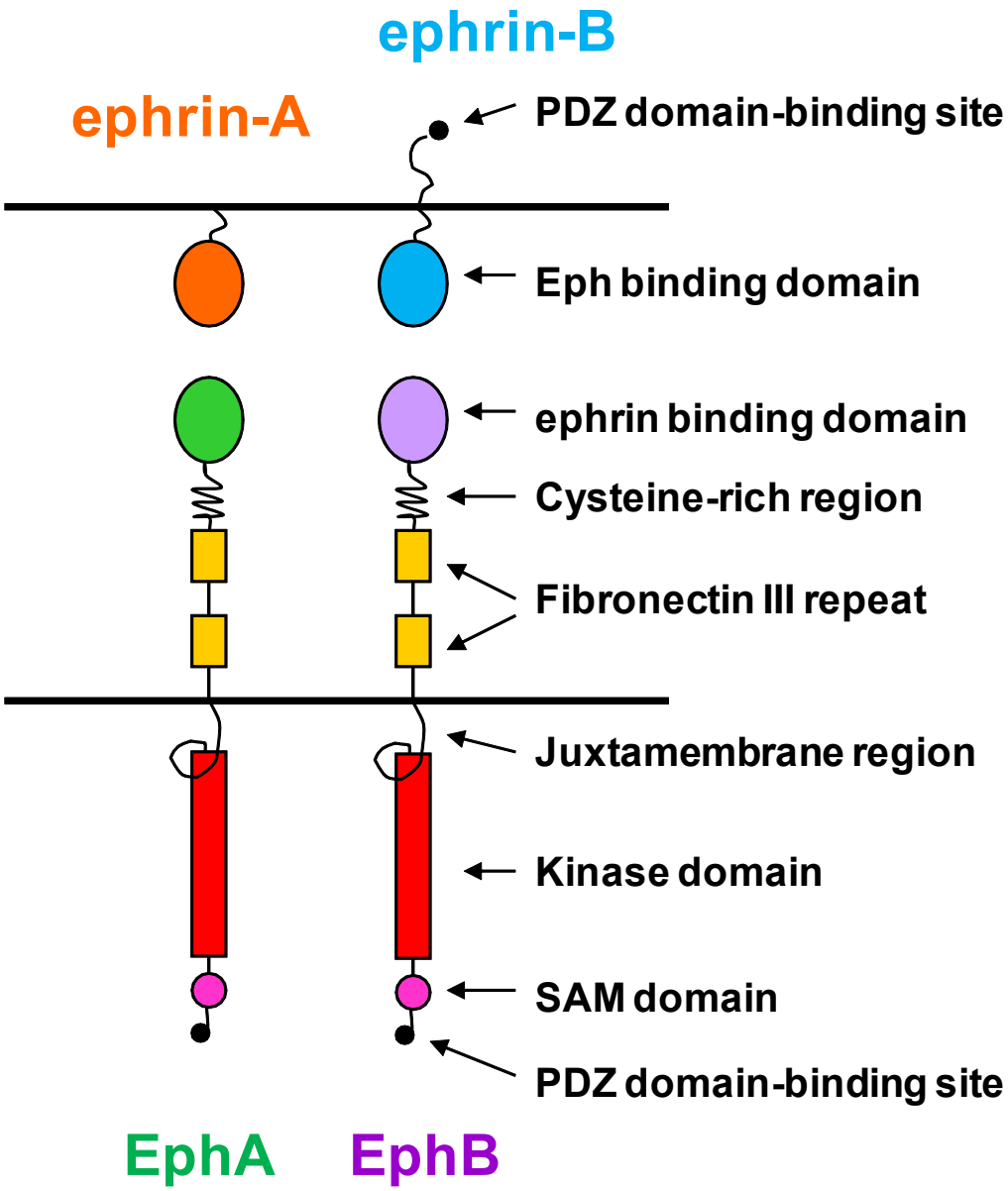

Figure 3.1. Eph receptor and ephrin ligand structure

Adapted from Yamaguchi and Pasquale (2004)

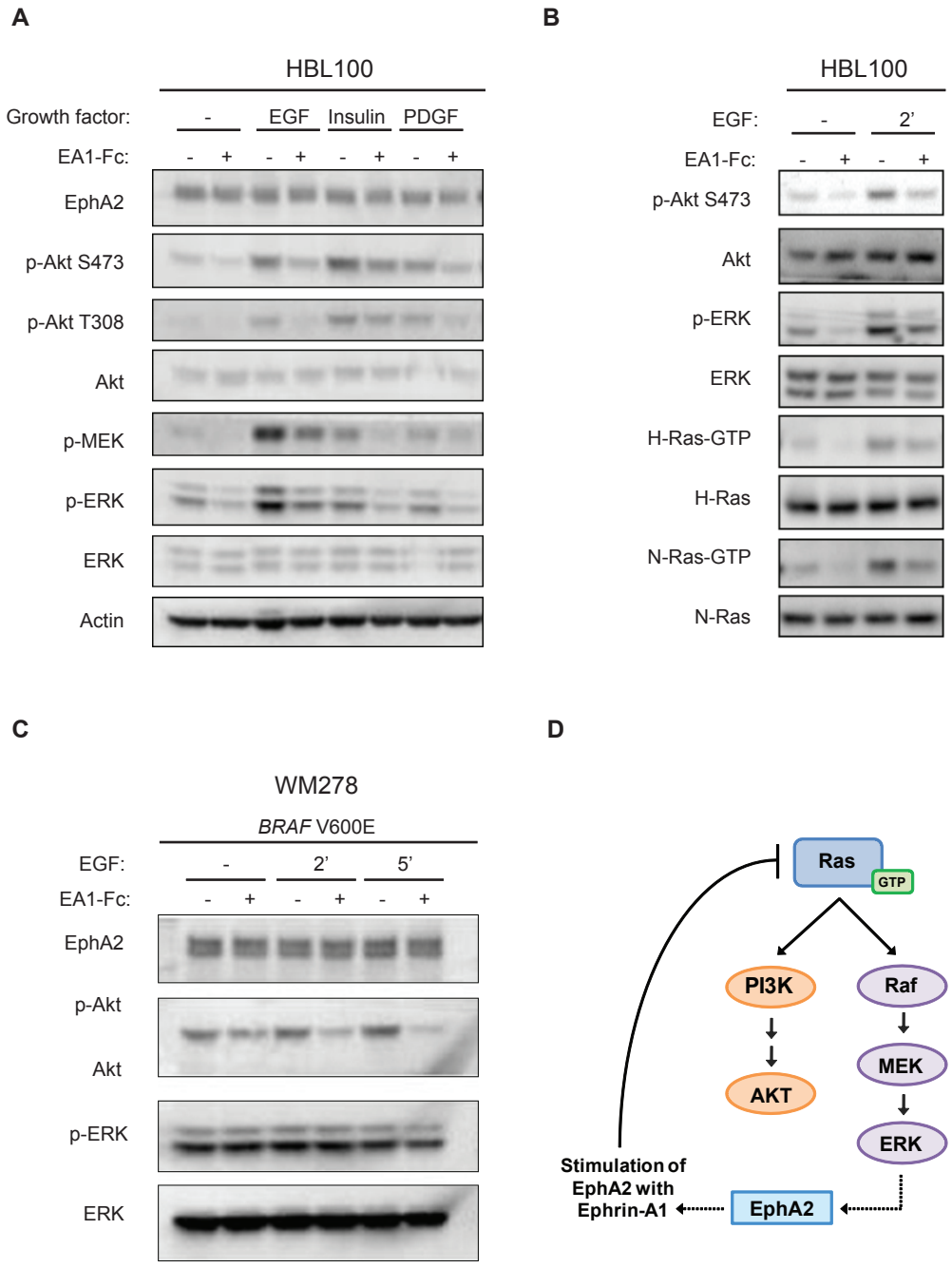

Figure 3.2. Stimulation with ephrin-A1-Fc suppresses Ras, MAPK and PI3K signaling

(A) Stimulation of HBL100 cells with ephrin-A1-Fc suppresses basal and growth factor-induced activation of MAPK and PI3K signaling. In all following experiments, phosphorylation of Akt at S473 and ERK at T204/Y202 serve as readouts of PI3K and MAPK pathway activity, respectively.
(B) Stimulation of HBL100 cells with ephrin-A1-Fc suppresses basal and EGF-induced Ras-GTP levels. Endogenous K-Ras protein levels were under the detection limit of the assay.
(C) Stimulation of WM278 melanoma cells with ephrin-A1-Fc suppresses MAPK, but not PI3K, signaling.
(D) Stimulation with ephrin-A1-Fc suppresses Ras activity and associated downstream pathways.

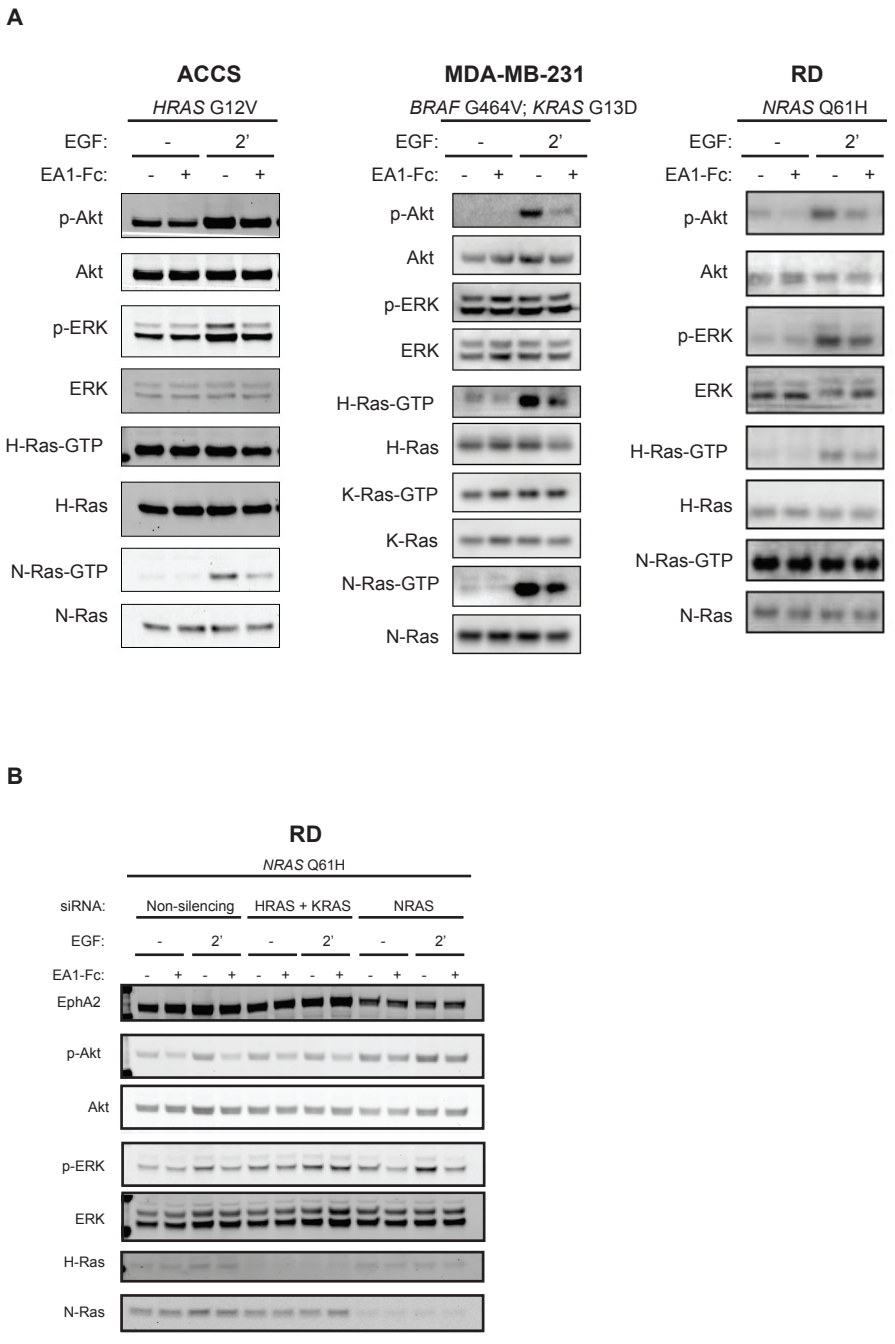

Figure 3.3. Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling in cancer cell lines harboring oncogenic *RAS* mutations

(A) Stimulation with ephrin-A1 suppresses the growth factor-induced activation of MAPK and PI3K signaling, which correlates with a reduction in GTP-loading on the wild-type, but not oncogenic, Ras isoforms. Endogenous K-Ras levels were below the detection limit of the assay for the ACCS and RD cell lines.
(B) Wild-type Ras isoforms are required to mediate suppression of MAPK, but not PI3K, pathway activity by ephrin-A1-Fc

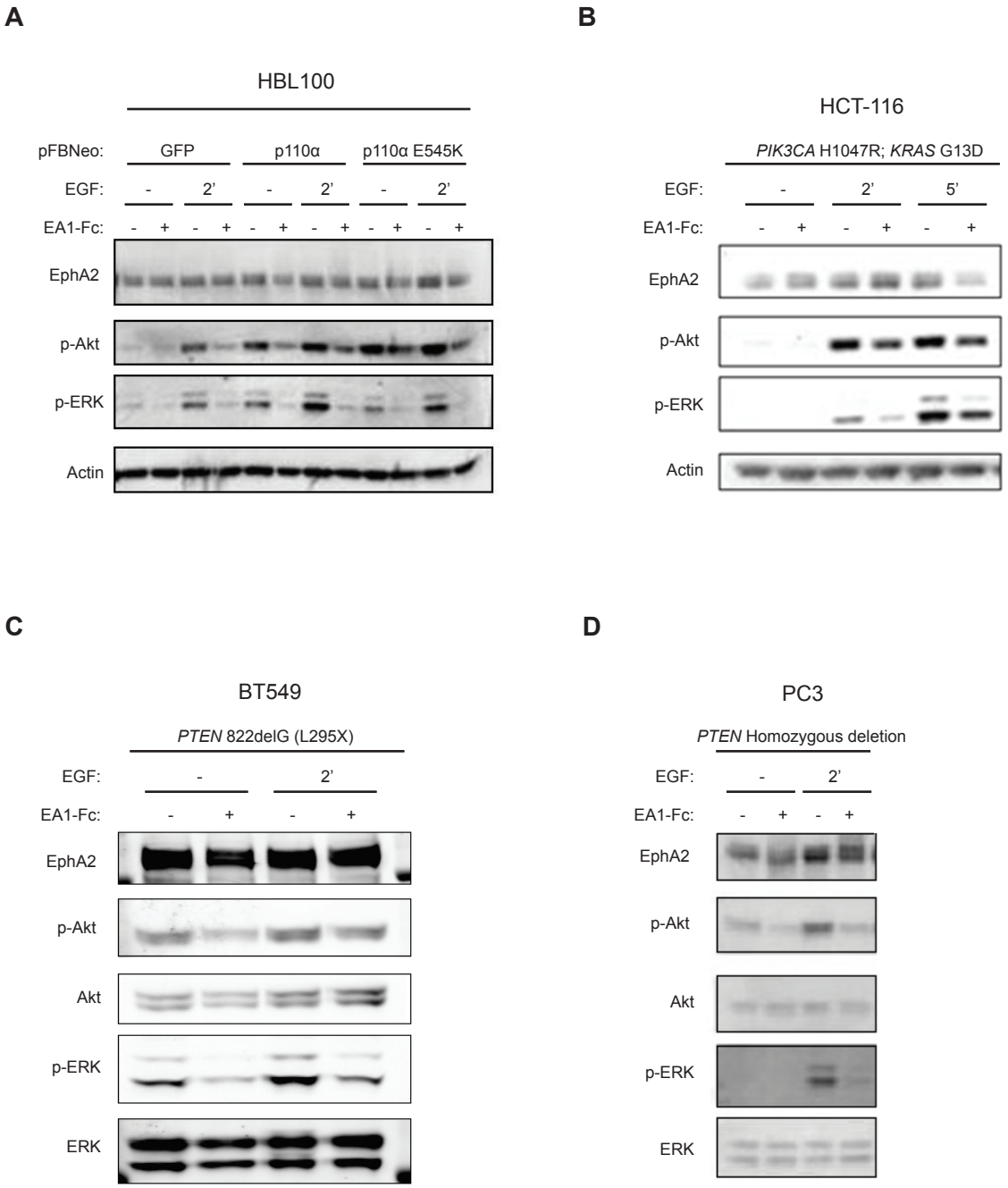

Figure 3.4. Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling in cancer cell lines with endogenous PI3K pathway mutations

(A) Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling downstream of ectopic wild-type or oncogenic p110α
(B) Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling downstream of endogenous oncogenic p110α
(C) PTEN is not required to mediate the suppression of PI3K signaling by ephrin-A1-Fc

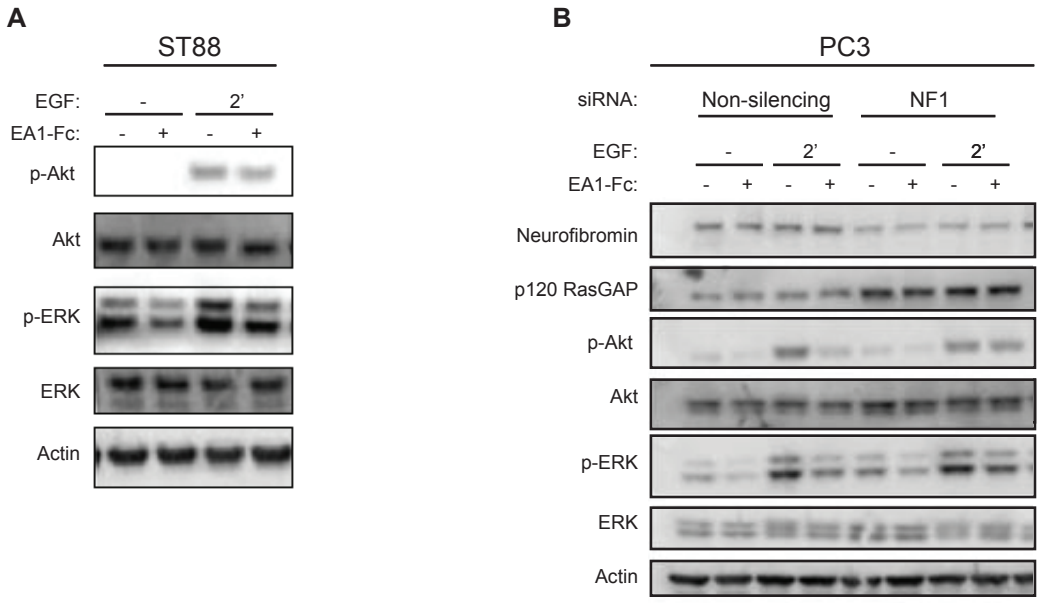

Figure 3.5. Neurofibromin is not required to mediate the suppression of MAPK or PI3K signaling downstream of ephrin-A1-Fc stimulation

(A) Stimulation of *NF1*-deficient ST88 cells with ephrin-A1-Fc suppresses MAPK and PI3K signaling
(B) Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling in cells depleted of neurofibromin by siRNA

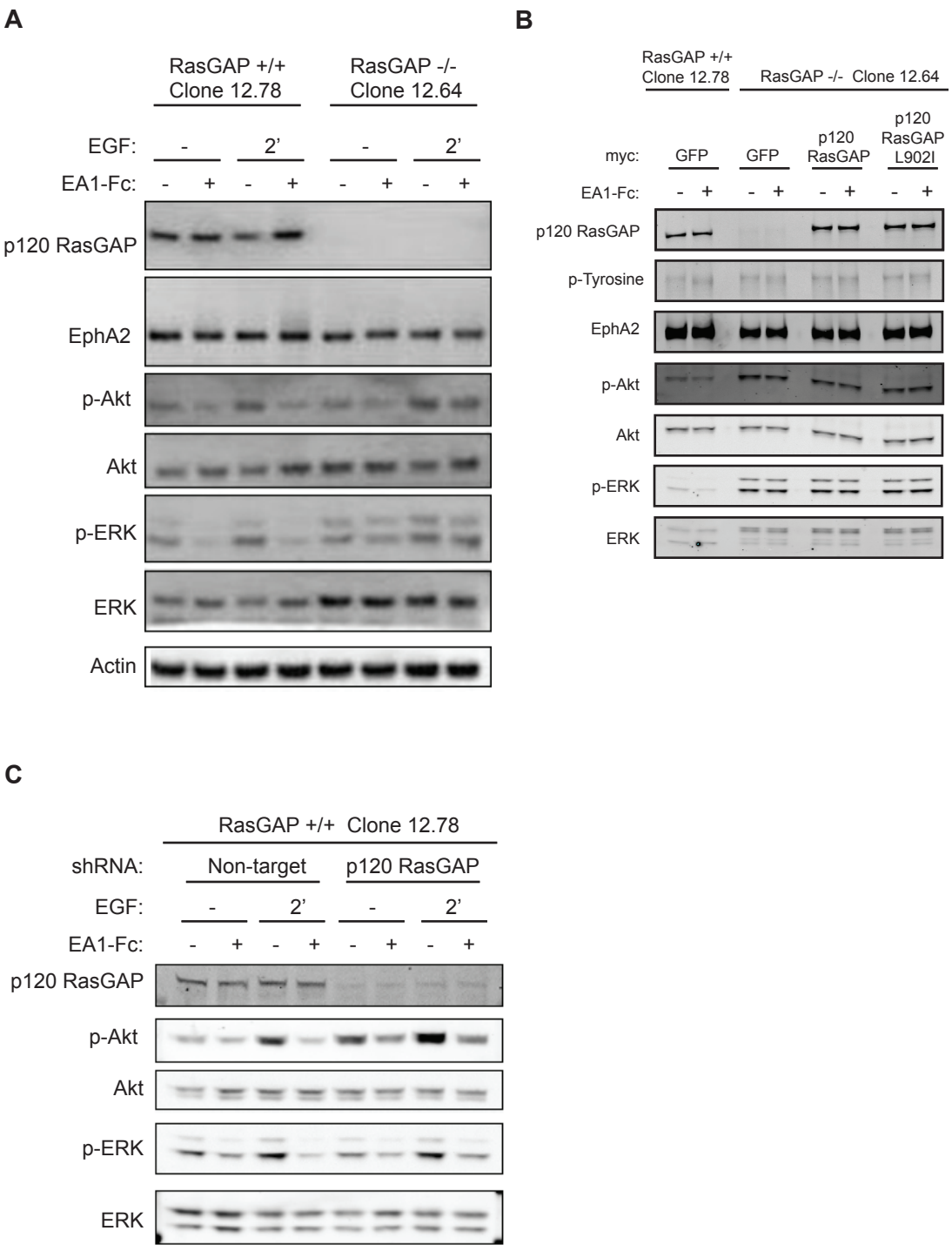

Figure 3.6. Stimulation with ephrin-A1-Fc does not suppress MAPK or PI3K signaling in p120 RasGAP-/- MEFs

(A) Clonal p120 RasGAP+/+ and p120 RasGAP-/- MEFs display differential sensitivity to ephrin-A1-Fc
(B) Reconstitution of p120 RasGAP-/- MEFs with ectopic p120 RasGAP does not rescue the ability of ephrin-A1-Fc stimulation to suppress MAPK or PI3K signaling
(C) Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling in p120 RasGAP+/+ MEFs depleted of p120 RasGAP by siRNA

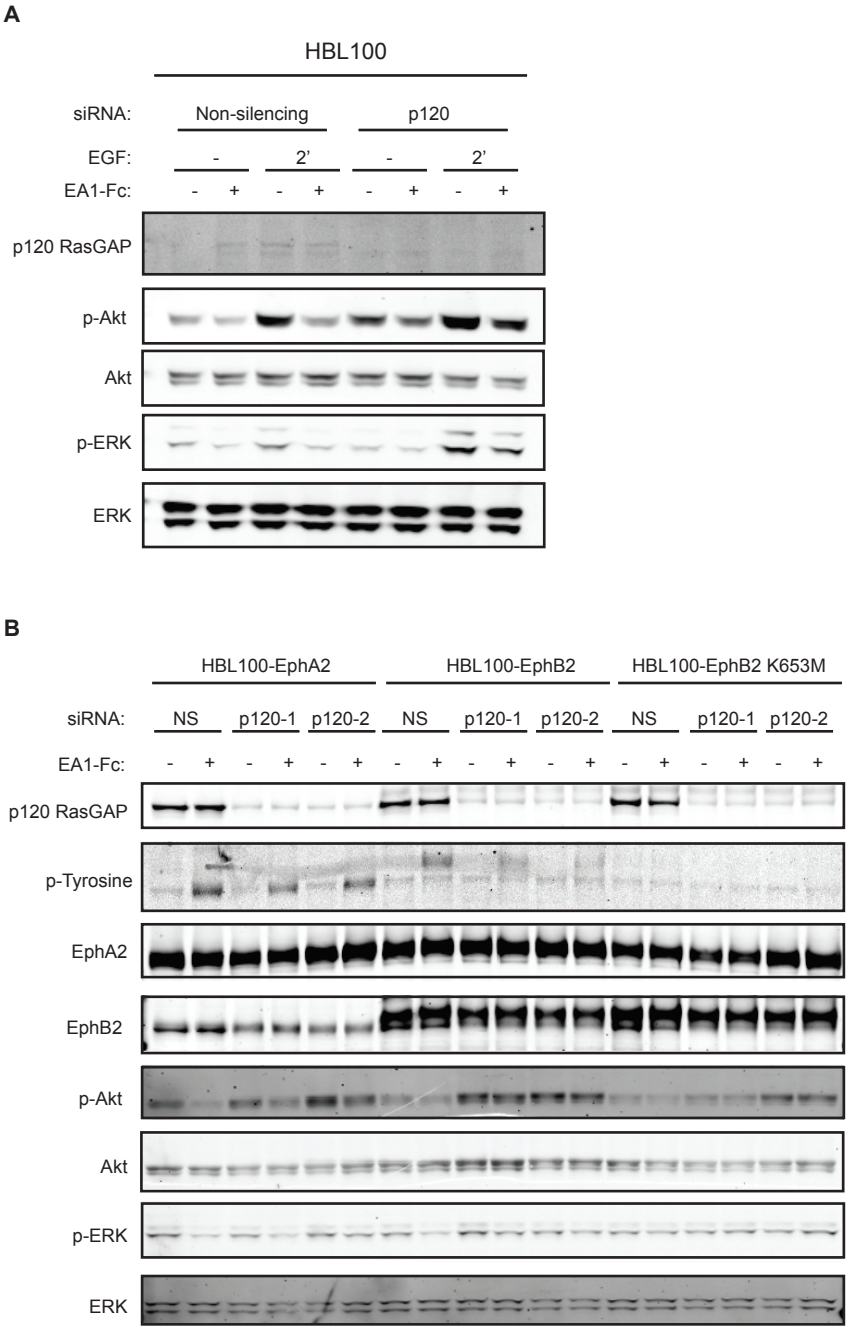

Figure 3.7. RNAi experiments do not conclusively support a role for p120 RasGAP in mediating the suppression of MAPK or PI3K signaling downstream of ephrin-A1-Fc stimulation

(A) Stimulation with ephrin-A1-Fc suppresses MAPK and PI3K signaling in HBL100 cells depleted of p120 RasGAP by siRNA
(B) siRNA-mediated depletion of p120 RasGAP blocks the attenuation of MAPK and PI3K signaling downstream of stimulation with ephrin-B1-Fc

CHAPTER 4.

A CRITICAL ROLE FOR WILD-TYPE RAS SIGNALING IN CANCER CELLS WITH ONCOGENIC RAS MUTATIONS

4.1. INTRODUCTION

The Ras signal transduction pathway is critically important in regulating cell growth, proliferation and survival and its aberrant hyperactivation has been shown to have a causal role in human cancer (Karnoub and Weinberg, 2008; Schubbert et al., 2007). Ras is a small G protein which cycles between an inactive GDP-bound state and an active GTP-bound state. When receptor tyrosine kinases at the cell surface are activated, guanine nucleotide exchange factors (GEFs) promote the exchange of bound GDP for GTP on Ras. In its GTP-bound state, Ras is able to interact with downstream effector proteins and activate a number of signaling pathways important for cell growth, including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, among others. To maintain a steady state of Ras signaling, GTPase activating proteins (GAPs) accelerate the hydrolysis of bound GTP to GDP on Ras, thereby dampening Ras activity. Ras is therefore able to act as a sensor of extracellular growth cues by cycling between an active GTP-bound state and an inactive GDP-bound state to ensure that signaling output through downstream effector pathways are of the appropriate intensity and duration.

Mutation of one of three canonical Ras genes - *HRAS*, *KRAS* or *NRAS* - is observed in an estimated 30% of human cancers. These activating point mutations lock Ras in an active GTP-bound state by rendering it insensitive the action of GAPs, allowing Ras to signal persistently to downstream effector pathways and promote the uncontrolled proliferation and survival of cancer cells. In cancers for which there is no Ras mutation, increased signaling through the pathway is achieved by amplification or activation of upstream receptor tyrosine kinases, loss-of-function mutation of negative regulators such as *NF1*, or via activating mutation of downstream effectors including *BRAF* and *PIK3CA*, illustrating the importance of high Ras pathway activity in the pathogenesis of cancer.

The oncogenic Ras isoform is thought to be primarily responsible for mediating the activation of downstream effector pathways, although the two wild-type Ras isoforms remain functional. In

fact, the signaling contribution of the wild-type Ras isoforms in this context has largely been discounted and unexamined. We were therefore surprised to discover that ephrin stimulation suppresses MAPK and PI3K signaling in cells that express activated alleles of *RAS*, and does so via the down-regulation of wild-type Ras activity (Chapter 3). This suggests that the wild-type Ras isoforms do indeed contribute to MAPK and PI3K signaling output in cancer cells with oncogenic *RAS* mutations.

In light of these findings, this study aims to determine whether the activation of MAPK and PI3K signaling is differentially regulated by wild-type and oncogenic Ras. The ability of growth factors to modulate wild-type and oncogenic Ras signaling will be investigated in detail. In addition, the role of wild-type and oncogenic Ras in maintaining basal steady-state levels of MAPK and PI3K signaling will also be examined. Finally, the requirement for wild-type and oncogenic Ras signaling in sustaining cell growth will be addressed.

4.2. RESULTS

4.2.i. Cancer cells with oncogenic RAS mutations are growth factor dependent

Growth factor receptors integrate external mitogenic signals to regulate cell growth and proliferation. Growth factor independence is one of the classic hallmarks of cancer, and can be achieved by constitutive activation or elevated expression of receptor tyrosine kinases or key regulatory proteins, such as Ras (Hanahan and Weinberg, 2011; Pedraza-Farina, 2006). However, the withdrawal of serum from culture medium significantly impedes the growth of cancer cell lines with endogenous oncogenic *RAS* mutations, suggesting that growth factor signaling is indeed important in these cells (Figure 4.1 A). In addition, stable expression of ectopic oncogenic H-Ras in a cell line free of endogenous MAPK or PI3K pathway mutations is not sufficient to confer growth factor independence, lending further support to this notion (Figure 4.1 B).

Because oncogenic Ras is constitutively GTP-bound, it is thought that signaling to downstream effector pathways maximally activated in cancer cell lines harboring oncogenic *RAS* mutations. However, stimulation with the growth factor EGF further enhances MAPK and PI3K signaling in these cells (Figure 4.2). As expected, oncogenic Ras is constitutively GTP-bound regardless of serum starvation or EGF stimulation. However, EGF stimulation results in an increase in Akt and ERK phosphorylation and a corresponding increase in GTP-loading on the wild-type Ras isoforms. These results demonstrate that the integrity of growth factor signaling is indeed intact in cancer cell lines harboring oncogenic *RAS* mutations.

4.2.ii. Wild-type Ras mediates growth factor-induced activation of MAPK and PI3K signaling, whereas oncogenic Ras maintains basal signaling

The results discussed above suggest that growth factor signaling might be mediated via the modulation of wild-type Ras activity. To formally test this, cells were transfected with siRNA to specifically silence the expression of either the oncogenic *RAS* isoform or the two wild-type *RAS* isoforms (Figure 4.3). For the ease of data interpretation, the majority of cell lines used in this study harbor homozygous *RAS* mutations (Table 2.2). siRNA-mediated depletion of the wild-type Ras isoforms significantly reduces the EGF-induced phosphorylation of ERK and Akt, without significantly altering EGFR phosphorylation, with respect to the control. Additionally, a similar pattern of phosphorylation is observed at the level of MEK and Raf-1. These results demonstrate that the wild-type Ras isoforms are required for growth factor-induced activation of MAPK and PI3K signaling in cancer cell lines with oncogenic *RAS* mutations. Importantly, these results (as well as the results presented below) have been confirmed in different cell lines using several unique siRNA sequences (data not shown).

In contrast, oncogenic Ras appears to be critical in maintaining the basal levels of MAPK and PI3K signaling. siRNA-mediated depletion of oncogenic Ras results in a slight but reproducible reduction in the basal levels of ERK phosphorylation (Figure 4.3). Thus, wild-type and oncogenic Ras differentially regulate MAPK and PI3K signaling based on cellular context: oncogenic Ras

maintains steady-state signaling, whereas the acute activation of wild-type Ras activity by growth factors enhances downstream signaling.

4.2.iii. Wild-type Ras antagonizes oncogenic Ras signaling in serum starved cells

In some cases, siRNA-mediated depletion of the wild-type Ras isoforms results in an increase in basal ERK phosphorylation relative to the control condition (Figure 4.3 B, C, E, F). A similar pattern of phosphorylation is observed at the level of MEK and Raf-1. This suggests that the wild-type Ras isoforms have an inhibitory effect on oncogenic Ras signaling. The mechanism by which this antagonism occurs is not fully understood. In some cell lines harboring oncogenic mutations in *KRAS*, siRNA-mediated depletion of the wild-type Ras isoforms results in a slight increase in total K-Ras protein levels (Figure 4.3 C, E, F). This increase in oncogenic K-Ras protein levels could in theory account for the slight increase in basal ERK phosphorylation by elevating the total cellular pool of GTP-bound Ras. Alternatively, wild-type Ras, which is predominantly GDP-bound in serum starved conditions, might antagonize oncogenic Ras signaling through a novel mechanism.

4.2.iv. Reciprocal regulation of RAS isoform expression

The relative levels of *HRAS*, *KRAS* and *NRAS* expression were examined in cancer cell lines harboring oncogenic mutations in each of the three *RAS* isoforms. In each cell line examined, *KRAS* expression levels were lowest with respect to the other two *RAS* isoforms (Figure 4.4 A). The ACCS cell line, which harbors an oncogenic mutation in *HRAS*, expressed the highest levels *HRAS* with respect to the other cell lines examined. The same trend held true for the other cell line as well - the MIA Paca-2 cell line, which harbors an oncogenic mutation in *KRAS*, expressed the highest levels *KRAS*, whereas the RD cell line, which harbors an oncogenic mutation in *NRAS*, expressed the highest levels of *NRAS*.

Interestingly, in each cell line examined, siRNA-mediated depletion of the oncogenic Ras isoform increases the expression levels of the two wild-type Ras isoforms. Conversely, siRNA-mediated

silencing of the wild-type *RAS* isoforms increases the expression level of oncogenic *RAS*. These results suggest there is a complex interplay in the regulation of *RAS* isoform expression.

4.2.v. Oncogenic Ras rewires EGFR signaling

In addition to maintaining basal signaling, oncogenic Ras also rewires EGFR signaling. siRNA-mediated depletion of oncogenic Ras results in the robust phosphorylation of EGFR upon acute stimulation with its ligand EGF. The increase in EGFR phosphorylation correlates with enhanced phosphorylation of ERK and Akt (Figure 4.3).

To extend upon these findings, a timecourse study of EGF stimulation was performed in MIA PaCa-2 cells in which expression of oncogenic K-Ras was depleted by siRNA (Figure 4.5 A). siRNA-mediated depletion of oncogenic K-Ras sensitizes cells to EGFR signaling. In addition, phosphorylation of EGFR at the Y1068 residue – the site at which the Grb2 adaptor protein binds – is enhanced and sustained with respect to the control. Conversely, phosphorylation at the T669 residue is decreased with respect to the control. It has been reported that activated ERK directly phosphorylates EGFR at T669 to inactivate the receptor, through a mechanism involving EGFR trafficking and turnover (Gan et al., 2010; Lemmon and Schlessinger, 2010; Li et al., 2008). Thus, silencing oncogenic K-Ras expression enhances the acute phosphorylation of EGFR on activating residues (Y1068) and relieves the phosphorylation of inhibitory residues (T669).

Several reports indicate that MEK inhibition relieves a negative feedback loop to the PI3K pathway that is mediated by EGFR (Hoeflich et al., 2009; Mirzoeva et al., 2009; Sos et al., 2009; Yoon et al., 2009). It is possible that siRNA-mediated depletion of oncogenic Ras expression achieves the same effect by suppressing the basal levels of MAPK signaling. To extend upon these findings, a timecourse study of EGF stimulation was performed in MIA PaCa-2 cells treated with the MEK inhibitor U0126 (Figure 4.5 B). The results obtained were strikingly similar to the scenario in which oncogenic K-Ras expression was silenced: MEK inhibition results in a robust activation of EGFR and Akt signaling upon acute EGF stimulation. Importantly, phosphorylation

of EGFR at Y1068 is markedly enhanced while phosphorylation at T669 is significantly reduced with respect to the control. The results suggest that suppressing basal MAPK signaling in *RAS* mutant cells – by either MEK inhibition or siRNA-mediated depletion of oncogenic Ras – amplifies EGFR signaling.

Furthermore, ectopic expression of oncogenic Ras is sufficient to desensitize EGFR signaling (Figure 4.6). HBL100 cells stably expressing ectopic oncogenic H-Ras, K-Ras or N-Ras are less sensitive to EGF stimulation with respect to the GFP-expressing control (Figure 4.6 A). EGF-induced phosphorylation of EGFR Y1068 is significantly reduced in cells stably expressing the ectopic oncogenic Ras isoforms, and this correlates with a reduction in EGF-induced Akt and ERK phosphorylation. Similar results are obtained in HEK 293 cells transiently expressing ectopic oncogenic Ras (Figure 4.6 B). Importantly, expression of a constitutively active form of MEK2 achieves the same results (Figure 4.6 B), lending support to the hypothesis that high levels of basal MAPK activity rewires EGFR signaling.

4.2.vi. Cancer cells with oncogenic *RAS* mutations depend on both wild-type and oncogenic Ras for cell growth

The results described above present a novel role for wild-type Ras signaling in cancer cells with oncogenic *RAS* mutations. Importantly, cell proliferation analyses also uncovered a critical role for wild-type Ras signaling in the growth and proliferation of *RAS* mutant cancer cells. As predicted, siRNA-mediated depletion of oncogenic Ras significantly reduces the rate of cell proliferation (Figures 4.7, 4.8, 4.9). In addition, siRNA-mediated depletion of the wild-type Ras isoforms also reduces the rate of cell proliferation. In two of the three cell lines examined, depletion of wild-type Ras expression results in a more mild reduction in cell growth with respect to depletion of oncogenic Ras. These results highlight the unexpected importance of wild-type Ras signaling in the growth of cancer cell lines with oncogenic *RAS* mutations.

4.3. DISCUSSION

The aim of this work has been to understand the role of wild-type Ras signaling in cancer cells that harbor an oncogenic *RAS* mutation. It has long been thought that the activity of oncogenic Ras is dominant over wild-type Ras, and is predominantly responsible for the activation of downstream signaling pathways. However, the work presented here reveals a critical role for wild-type Ras signaling: the wild-type Ras isoforms can be activated by mitogenic growth cues and inactivated by intrinsic regulatory proteins, and the resultant changes in wild-type Ras activity in turn modulate the intensity of signaling to downstream pathways, despite the presence of oncogenic Ras (Figure 4.10). Additionally, this work demonstrates a critical role for wild-type Ras signaling in the proliferation of cancer cells expressing oncogenic *RAS*. This suggests that wild-type Ras proteins might serve as appropriate targets for therapeutic intervention in tumors with oncogenic *RAS* mutations. There are currently no effective therapeutic treatments available for cancers expressing oncogenic *RAS*, and efforts to develop drugs that specifically block the activity of oncogenic Ras have been unsuccessful. However, the discovery that wild-type Ras plays a major role in signaling in cancer cells with oncogenic *RAS* mutations suggests a novel strategy for treating these types of cancers, in which the activity of wild-type Ras is blocked in order to impede cell growth.

While wild-type Ras appears most critical for growth factor-induced signaling, this study establishes the importance of oncogenic Ras in maintaining the basal level of steady-state signaling. Furthermore, the data presented here uncover a role for oncogenic Ras in rewiring EGFR signaling, and are consistent with a model in which high levels of MAPK activity inhibit EGFR signaling. Clinical studies indicate that oncogenic *KRAS* mutations are predictive of resistance to EGFR-based therapies (Wheeler et al., 2010). One mechanism by which oncogenic mutation in *KRAS* desensitizes cells to EGFR inhibitors might involve a complete rewiring of EGFR signaling dynamics. Suppression of MAPK signaling – whether by silencing of oncogenic *KRAS* expression or by pharmacological inhibition of MEK kinase activity – resensitizes EGFR

signaling and primes the receptor for activation or inhibition. In support of this, recent studies indicate that dual treatment with MEK and EGFR inhibitors synergistically inhibits the growth of cancer cells harboring oncogenic *KRAS* mutations and significantly reduces the IC_{50} for the EGFR inhibitor (Yoon et al., 2009; Yoon et al., 2010). Taken together, these data suggest dual targeting of EGFR and the MAPK signaling pathway might be beneficial in cancers harboring oncogenic *RAS* mutations. Importantly, this approach would concurrently block the activation of wild-type Ras signaling by EGFR while suppressing the basal levels of MAPK signaling sustained by oncogenic Ras.

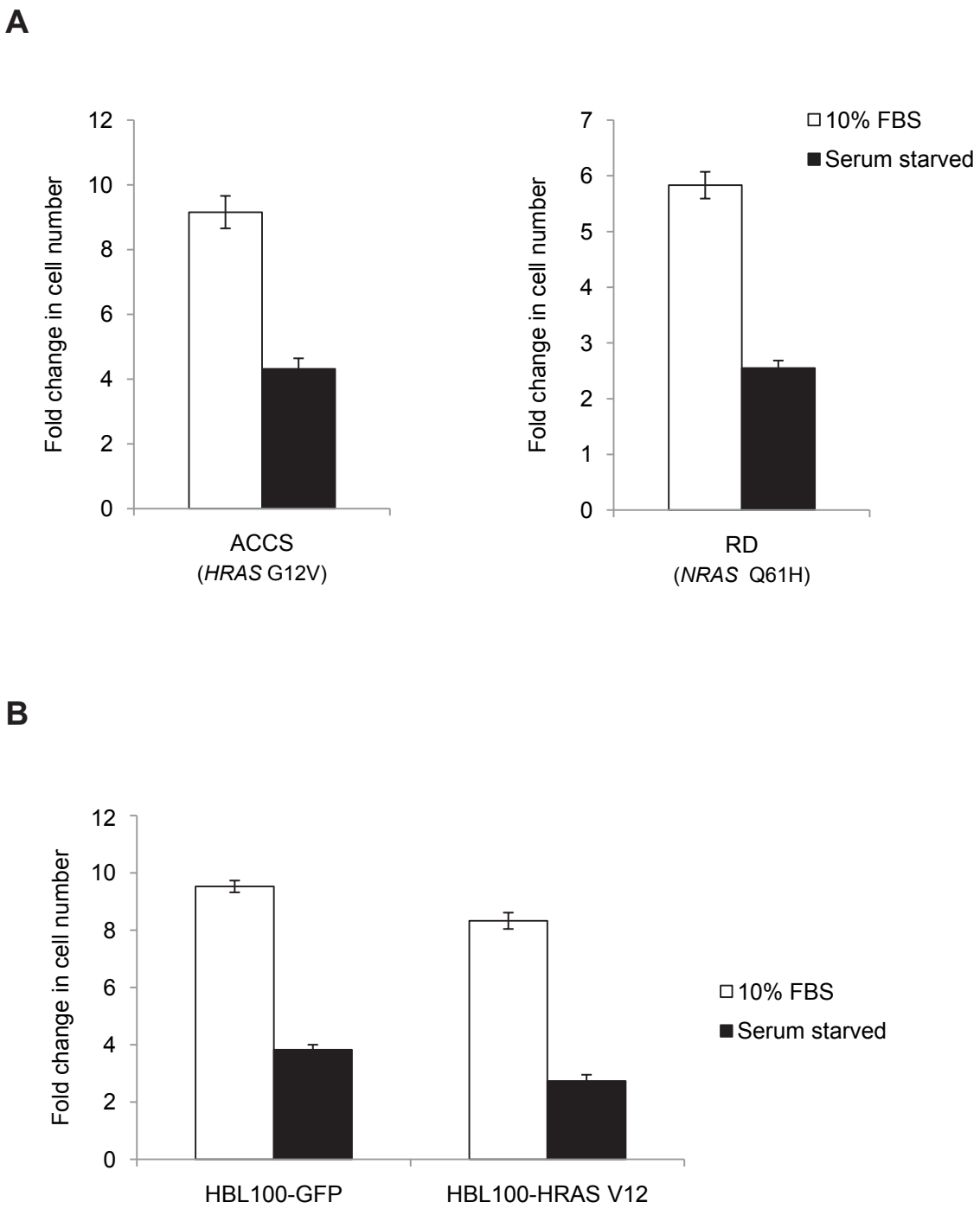

Figure 4.1. Cancer cells with oncogenic *RAS* mutations are growth factor dependent

(A) Serum starvation of cell lines with endogenous oncogenic *RAS* mutations impedes cell growth. Cancer cell lines were seeded in triplicate and placed in either serum-free or complete media the following day. Cells counts were obtained 24 hours post seeding and 72 hours post media change. Results are displayed as a fold increase in cell number relative to the initial cell count.
(B) Stable expression of oncogenic Ras does not confer growth factor independence HBL100 cells expressing either GFP or H-Ras V12 were subjected to similar serum starvation experiments as described in (A). The withdrawal of growth factors impedes cell growth to the same extent in both cell lines.

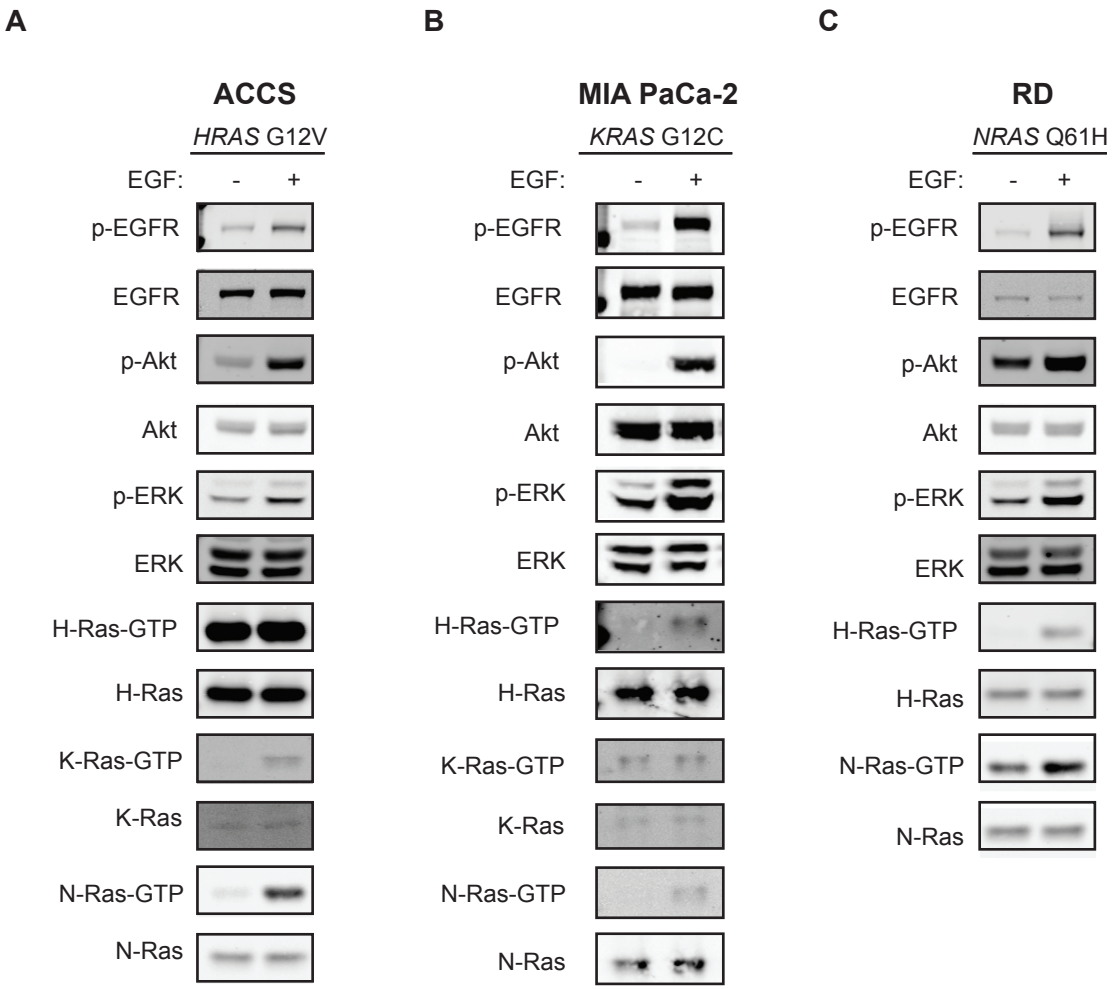

Figure 4.2. Growth factors enhance MAPK and PI3K signaling in cancer cells with oncogenic *RAS* mutations

Cancer cell lines with oncogenic mutations in *HRAS* (A), *KRAS* (B), and *NRAS* (C) were serum starved overnight before acute stimulation with EGF. Oncogenic Ras remains constitutively GTP-bound regardless of serum starvation or EGF stimulation. EGF stimulation results in an increase in Akt and ERK phosphorylation and a corresponding increase in GTP-loading on the wild-type Ras isoforms. K-Ras protein levels are below the detection limit of the assay in (C).

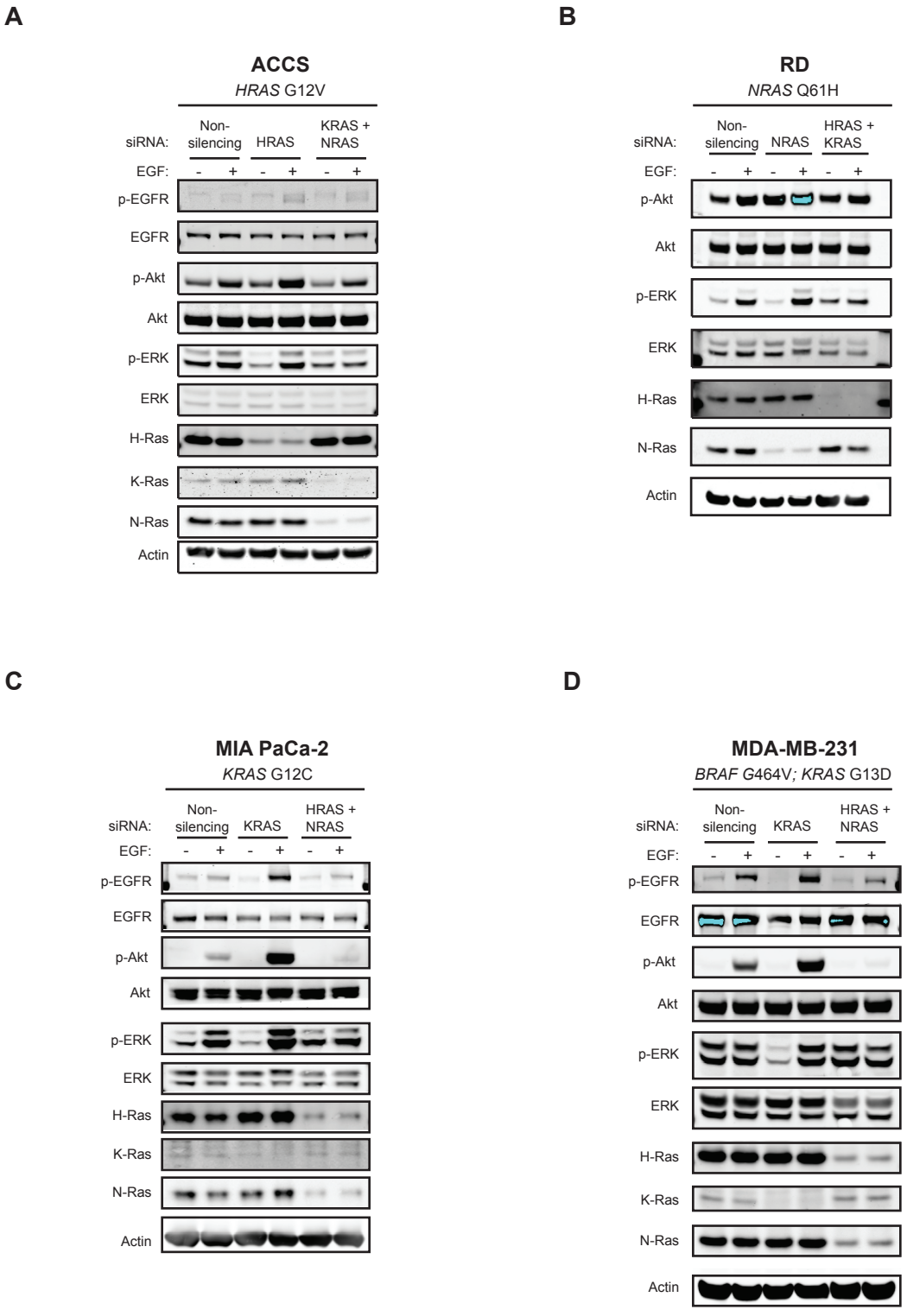

Figure 4.3. Wild-type Ras mediates growth factor-induced activation of MAPK and PI3K signaling, whereas oncogenic Ras maintains basal signaling

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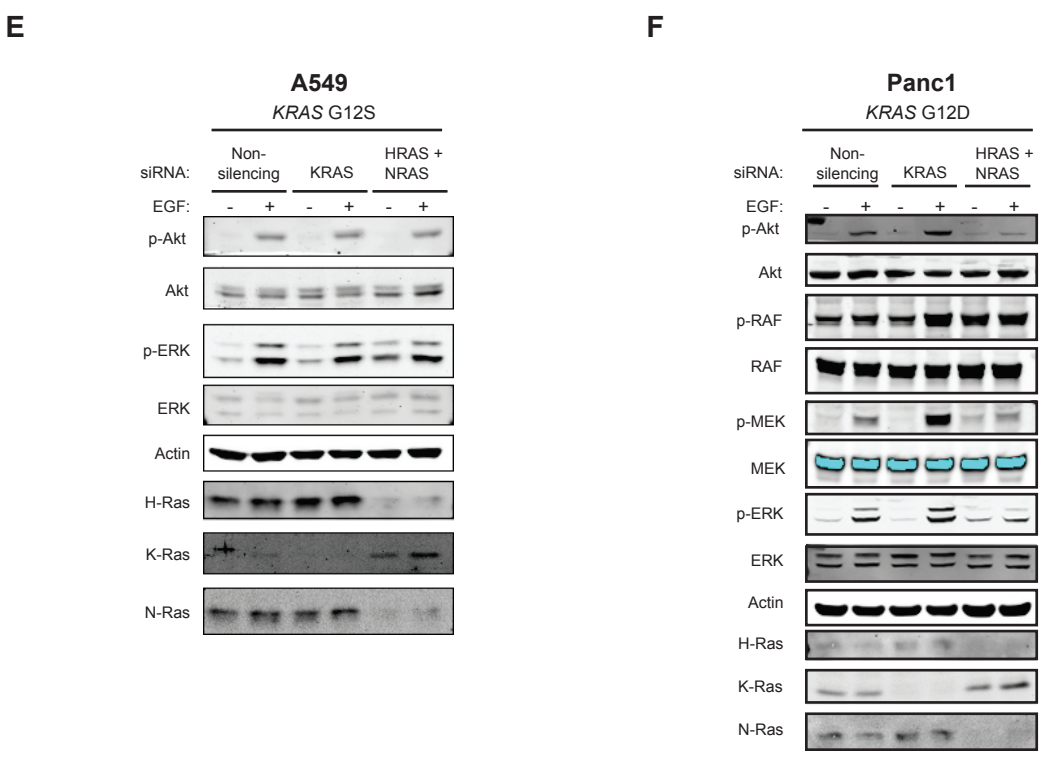


Figure 4.3. Wild-type Ras mediates growth factor-induced activation of MAPK and PI3K signaling, whereas oncogenic Ras maintains basal signaling

Cells were transfected with the indicated siRNAs and serum starved overnight before acute stimulation with EGF. siRNA-mediated depletion of the oncogenic Ras isoform decreases basal levels of ERK phosphorylation and sensitizes cells to EGFR signaling. siRNA-mediated depletion of the wild-type Ras isoforms reduces the EGF-induced phosphorylation of ERK and Akt, without significantly altering EGFR phosphorylation, with respect to the control. In some cases, siRNA-mediated depletion of the wild-type Ras isoforms increases basal ERK phosphorylation (B, C, E, F). Additionally, in some cell lines with oncogenic mutations in *KRAS*, siRNA-mediated depletion of the wild-type Ras isoforms slightly increases the total K-Ras protein levels (C, E, F). K-Ras protein levels are below the detection limit of the assay in (B).

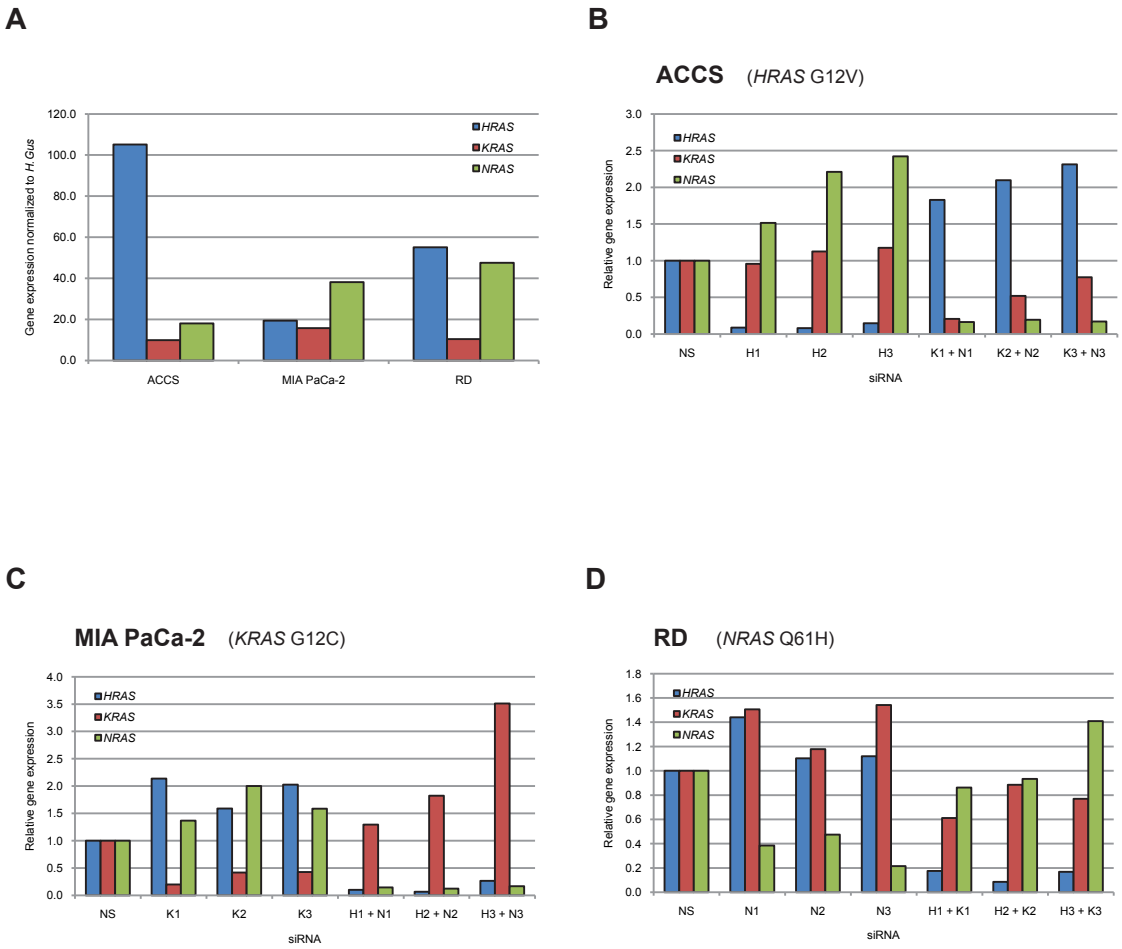


Figure 4.4. Reciprocal regulation of *RAS* isoform expression

qRT-PCR was performed on reverse-transcribed RNA derived from cancer cells transfected with various combinations of siRNAs targeting either oncogenic or wild-type *RAS*. Expression levels were normalized to the housekeeping gene *H.GUS*, and in (B, C, D) are displayed as as values relative to the non-silencing control siRNA condition. NS: non-silencing siRNA control. H1, H2, H3: three independent siRNAs targeting *HRAS*. K1, K2, K3: three independent siRNAs targeting *KRAS*. N1, N2, N3: three independent siRNAs targeting *NRAS*.

(A) Relative expression levels of *HRAS*, *KRAS* and *NRAS* are compared among three cancer cell lines with mutations in each of the three *RAS* isoforms. Samples represent cells transfected with non-silencing control siRNA.

(B, C, D) siRNA-mediated silencing of the oncogenic *RAS* isoform increases the expression levels of the two wild-type *RAS* isoforms, and vice versa.

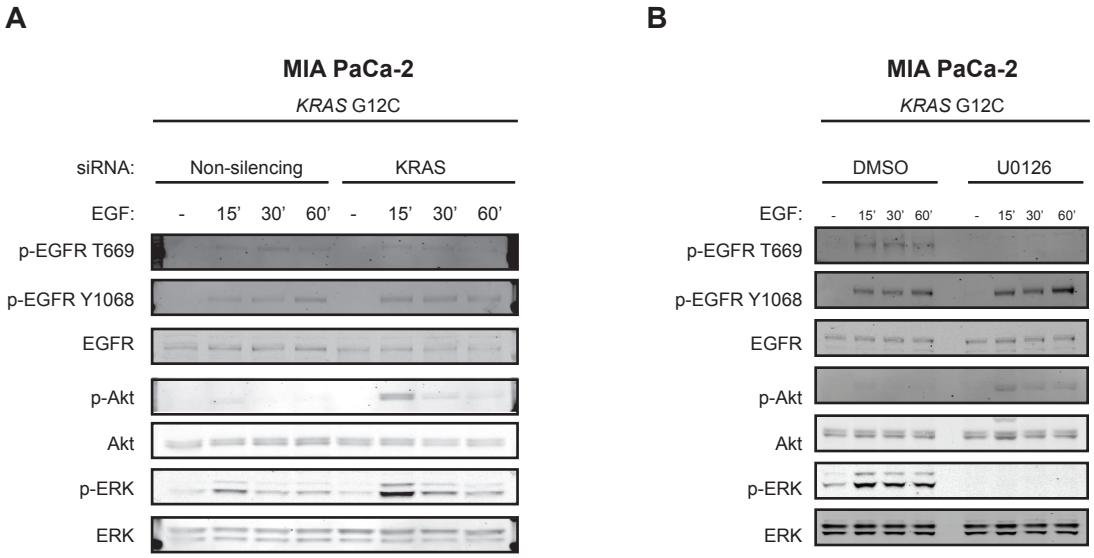


Figure 4.5. Suppression of basal MAPK pathway activity sensitizes *RAS* mutant cancer cells to EGFR signaling

(A) siRNA-mediated depletion of K-Ras in the MIA PaCa-2 cell line slightly reduces basal ERK phosphorylation and sensitizes cells to acute stimulation with EGF.

(B) Treatment with the MEK inhibitor U0126 also reduces basal ERK phosphorylation and sensitizes cells to acute stimulation with EGF.

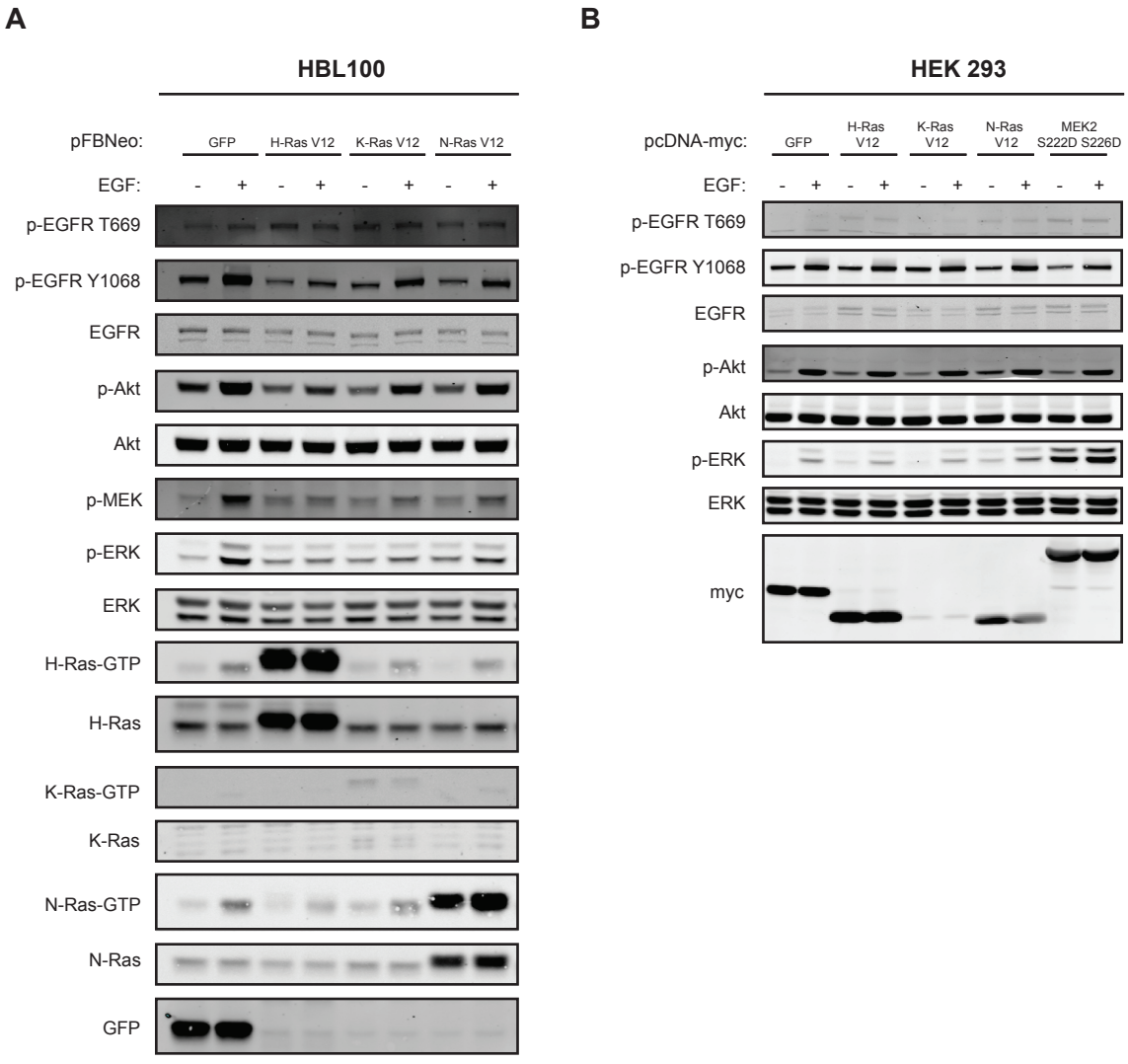
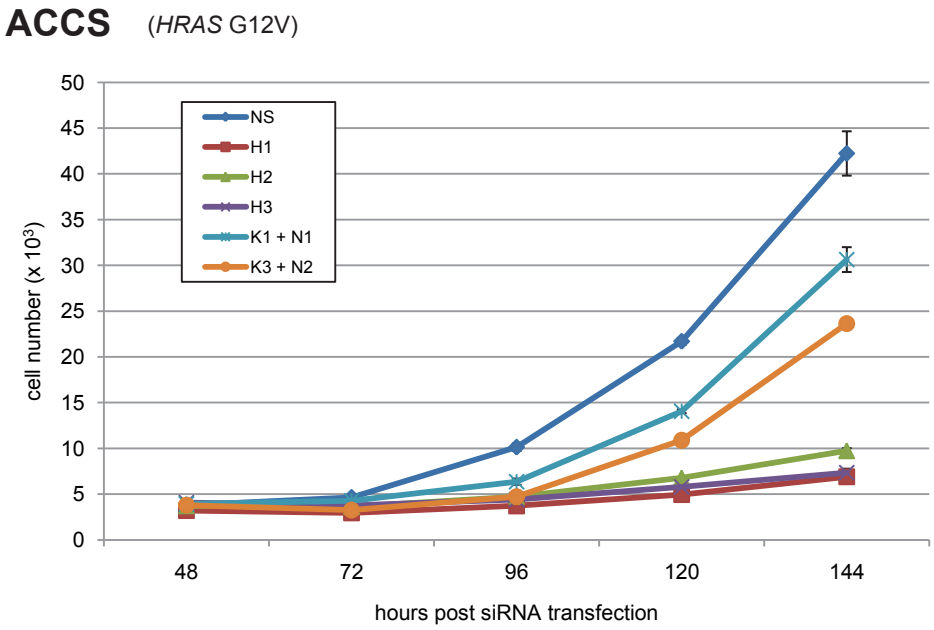


Figure 4.6. Ectopic expression of oncogenic Ras attenuates EGFR signaling

(A) HBL100 cells stably expressing GFP or oncogenic H-Ras, K-Ras or N-Ras were serum starved overnight before acute stimulation with EGF. Ectopic expression of oncogenic Ras slightly increases basal ERK phoshporylation and desensitizes cells to EGFR signaling. Ras-GTP assays confirm that ectopic oncogenic Ras is constitutively GTP-bound.

(B) HEK 293 cells were transiently transfected with myc-tagged GFP or constitutively active forms of H-Ras, K-Ras, N-Ras or MEK2 and serum starved overnight before acute stimulation with EGF. In cells expressing constitutively active forms of Ras and MEK, basal ERK levels are elevated while EGF-induced phosphorylation levels of ERK are reduced with respect to the GFP control. Note that the low level of basal ERK phosphorylation in cells expressing ectopic K-Ras V12 is likely due to the low level of ectopic protein expression.



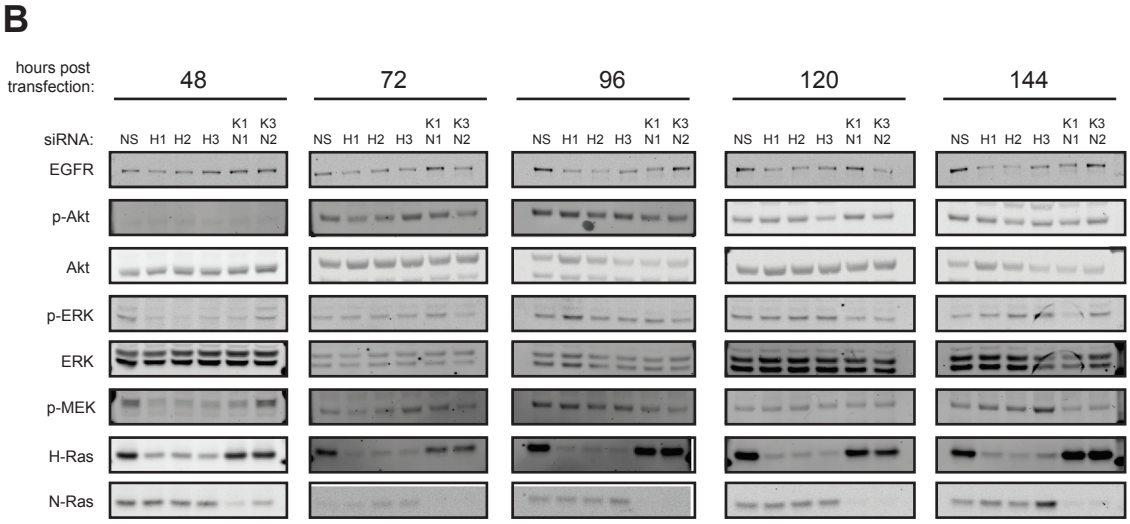


Figure 4.7. siRNA-mediated depletion of oncogenic or wild-type Ras impedes the growth of ACCS bladder cancer cells

(A) Cells were transfected with various combinations of siRNAs targeting either oncogenic or wild-type *RAS*. Two days after transfection, cells were counted and seeded in triplicate at equal densities in complete medium. Accurate cell counts were obtained every 24 hours using a Coulter particle counter. An initial cell count was obtained at the time of seeding to confirm equal seeding conditions.

(B) Samples were harvested in parallel to the cell proliferation analysis for immunoblot analysis. K-Ras protein levels are below the detection limit of the assay.

NS: Non-silencing siRNA control. H1, H2, H3: three independent siRNAs targeting *HRAS*. K1, K3: two independent siRNAs targeting *KRAS*. N1, N2: two independent siRNAs targeting *NRAS*.

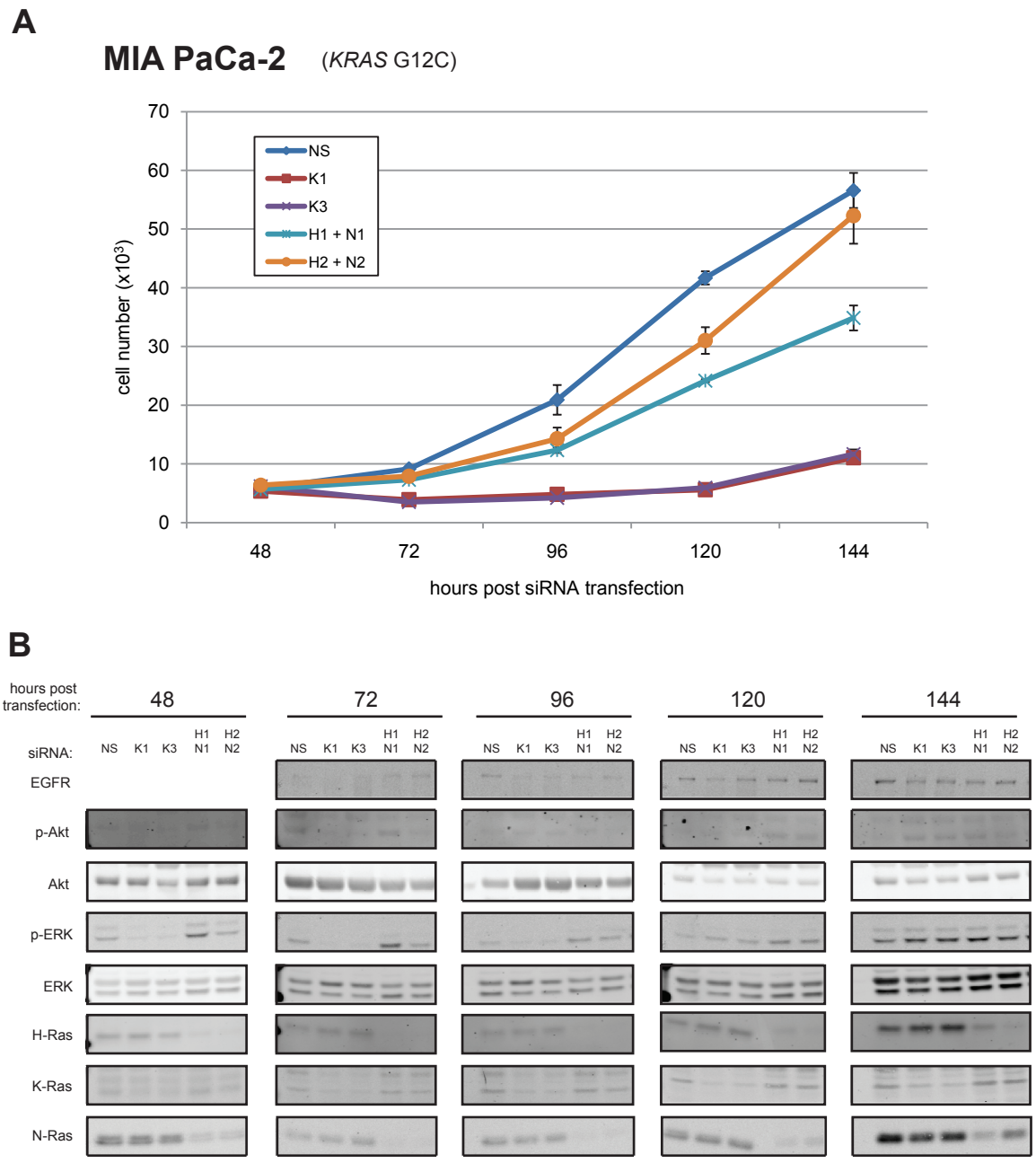


Figure 4.8. siRNA-mediated depletion of oncogenic or wild-type Ras impedes the growth of MIA PaCa-2 pancreatic cancer cells

(A) Cells were transfected with various combinations of siRNAs targeting either oncogenic or wild-type *RAS*. Two days after transfection, cells were counted and seeded in triplicate at equal densities in complete medium. Accurate cell counts were obtained every 24 hours using a Coulter particle counter. An initial cell count was obtained at the time of seeding to confirm equal seeding conditions.

(B) Samples were harvested in parallel to the cell proliferation analysis for immunoblot analysis.

NS: Non-silencing siRNA control. H1, H2: two independent siRNAs targeting *HRAS*. K1, K3: two independent siRNAs targeting *KRAS*. N1, N2: two independent siRNAs targeting *NRAS*.

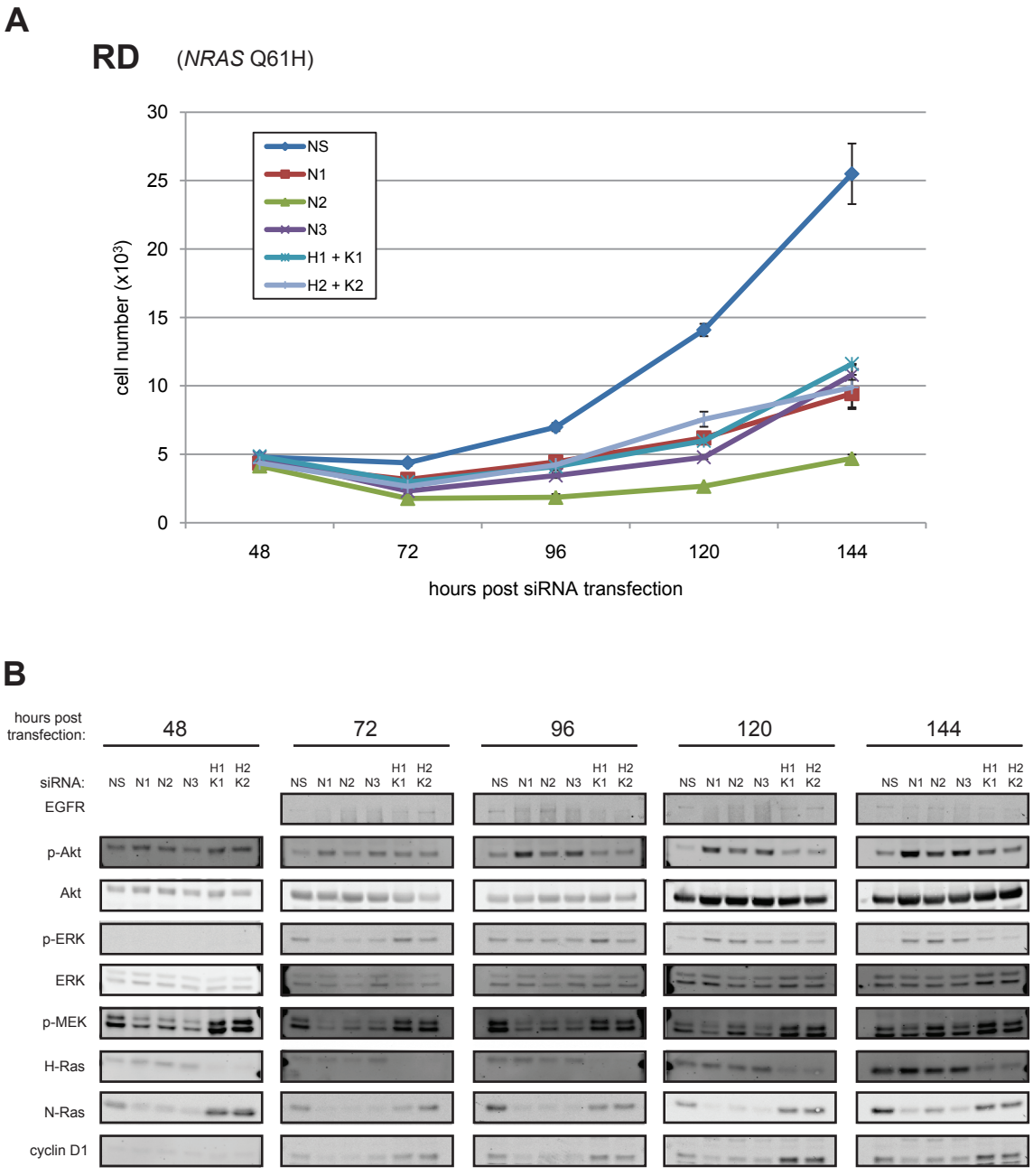


Figure 4.9. siRNA-mediated depletion of oncogenic or wild-type Ras impedes the growth of RD rhabdomyosarcoma cells

(A) Cells were transfected with various combinations of siRNAs targeting either oncogenic or wild-type *RAS*. Two days after transfection, cells were counted and seeded in triplicate at equal densities in complete medium. Accurate cell counts were obtained every 24 hours using a Coulter particle counter. An initial cell count was obtained at the time of seeding to confirm equal seeding conditions.

(B) Samples were harvested in parallel to the cell proliferation analysis for immunoblot analysis. K-Ras protein levels are below the detection limit of the assay.

NS: Non-silencing siRNA control. H1, H2: two independent siRNAs targeting *HRAS*. K1, K2: two independent siRNAs targeting *KRAS*. N1, N2, N3: three independent siRNAs targeting *NRAS*.

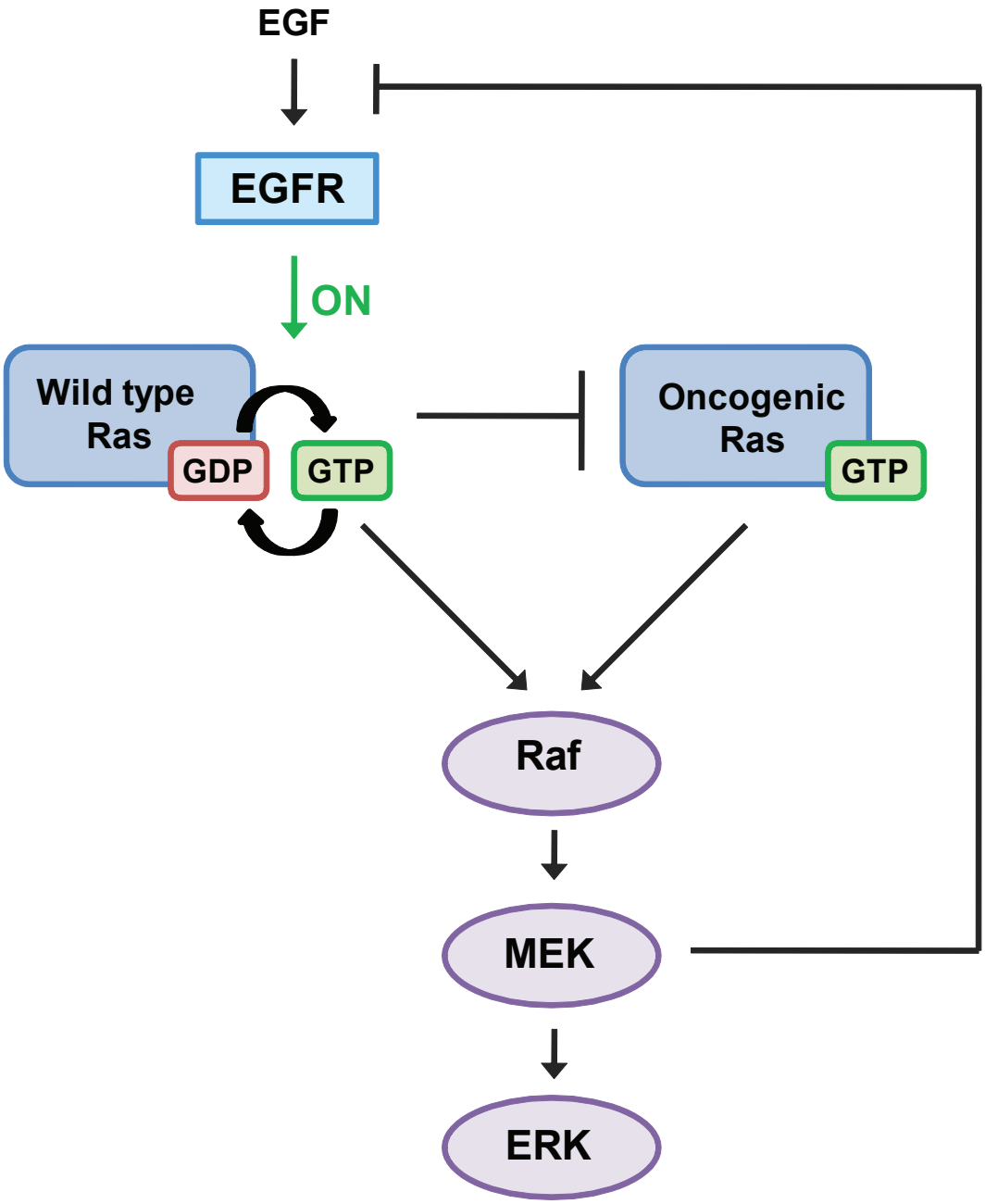


Figure 4.10. Regulation of signaling by wild-type and oncogenic Ras

Oncogenic Ras is constitutively GTP-bound and signals persistently to downstream effector pathways. Wild-type Ras cycles between an inactive GDP-bound form and an active GTP-bound form. Oncogenic Ras is primarily responsible for maintaining basal signaling. Wild-type Ras is required for maintaining growth factor-induced signaling. In the basal state, wild-type Ras, which is predominantly GDP-bound, inhibits oncogenic Ras signaling through a mechanism that is not yet fully understood. High levels of basal MAPK signaling, often observed in cells with oncogenic *RAS* mutations, dampens EGFR signaling through a negative feedback loop. Suppression of basal MAPK signaling in these cells - via MEK inhbition or via siRNA-mediated depletion of oncogenic Ras - resensitizes cells to EGFR signaling.

CHAPTER 5.

CONCLUDING REMARKS

5.1. FUTURE DIRECTIONS REGARDING EPHA2 SIGNALING

5.1.i. Caveats and considerations

Several experimental caveats and limitations regarding the experimental tools used to study EphA2 signaling should be taken into consideration, and are discussed in further detail below.

Ephrin-A1-Fc promiscuously interacts with and activates all EphA family kinases. As such, signaling changes induced by ephrin-A1-Fc stimulation cannot be exclusively ascribed to EphA2 activation. Selective agonists of EphA2, including the YSA peptide and the monoclonal antibody 1C1, are therefore preferable experimental tools (Jackson et al., 2008; Koolpe et al., 2002). Alternatively, as a control, siRNA-mediated depletion of EphA2 should demonstrate that the outcome of ephrin-A1-Fc stimulation is dependent on the expression of EphA2.

A second complication of the reagent is the fact that the Fc portion of ephrin-A1-Fc binds protein A and protein G with high affinity, posing complications when performing immunoprecipitation experiments with protein A or protein G resin. To circumvent this issue, immunoprecipitation antibodies could be covalently coupled to magnetic beads, thereby circumventing any issues regarding the background binding of ephrin-A1-Fc.

Identifying interacting partners of EphA2 has been a technical challenge due to the nature of receptor tyrosine kinase signaling, in which interactions with binding partners is often transient in nature. For example, although it has been well established that activated EphB2 recruits p120 RasGAP, we have been unable to demonstrate this interaction, even in a system in which ectopic EphB2 is overexpressed (data not shown). Future experiments could utilize a number of different techniques to enhance the probability of detecting an interaction of EphA2 or EphB2 with their respective binding partners. For example, cells could be chemically cross-linked prior to immunoprecipitation to aid in preserving weak or transient interactions. Alternatively, immunoprecipitation experiments performed with antibodies covalently coupled to magnetic beads would reduce background binding and assay time, and might therefore enhance the probability of detecting transient or low-affinity interactions.

5.1.ii. Mechanism of the attenuation of Ras and Akt signaling

It is still unclear exactly how activated EphA2 suppresses Ras and Akt signaling. The evidence presented in this work is consistent with reports suggesting that Ras and Akt are independently regulated downstream of EphA2 activation (Yang et al., 2011).

An unbiased proteomic approach might identify novel binding partners of EphA2 that could be involved in the suppression of Ras or Akt activity. Conditions should include wild-type EphA2 and a kinase-dead mutant (EphA2 K646M) as well as EphB2 and a kinase-dead mutant (EphB2 K653M). A C-terminal tag would likely be required since the ligand binding domain resides in the N-terminal extracellular region of Eph receptors. We have evidence demonstrating that EphA2 and EphB2 constructs containing tags at the C-termini retain the ability to suppress Ras and Akt signaling downstream of ephrin stimulation (Figure 3.7 B). Small scale immunoprecipitation experiments should be optimized beforehand to verify that known binding partners of EphA2 and EphB2 can be detected in immunoprecipitation complexes.

5.1.iii. Phenotypic consequence of ephrin-A1-Fc stimulation in cancer cell lines harboring oncogenic RAS mutations

Several studies have shown that the activation of EphA2 results in a reduction in cell proliferation, motility and invasiveness *in vitro*, impaired three-dimensional growth in agar and reconstituted basement membrane matrix, as well as decreased tumor growth *in vivo* (Duxbury et al., 2004b; Miao et al., 2000; Miao et al., 2009; Miao et al., 2003; Miao et al., 2005; Miao et al., 2001; Noblitt et al., 2004; Wykosky et al., 2005; Zelinski et al., 2001). However, an outstanding question is whether the activation of EphA2 might have similar consequences in cancer cells harboring oncogenic *RAS* mutations. Ephrin-A1-Fc stimulation has a more pronounced effect on attenuating growth factor-induced signaling than basal signaling in these cells (Figure 3.3). Phenotypic assays that measure a response to growth factor exposure might therefore be most

appropriate. In cells without endogenous *RAS* mutations, ephrin-A1-Fc stimulation inhibits growth factor-induced cell migration *in vitro*, as measured by wound healing and transwell migration assays (Miao et al., 2009; Parri et al., 2005). It would be interesting to repeat these experiments in a panel of cancer cell lines harboring oncogenic *RAS* mutations. Conditions in which either oncogenic Ras or the wild-type Ras isoforms are depleted by siRNA should be included. Based on the biochemical data, one might predict that depleting oncogenic Ras expression would enhance the ability of ephrin-A1-Fc stimulation to suppress cell migration *in vitro*, whereas silencing the expression of the wild-type Ras isoforms would diminish the effect. Control conditions should include treatment with MEK and PI3K inhibitors, both alone and in combination, prior to ephrin-A1-Fc stimulation. These experiments will determine whether the ephrin-mediated attenuation of wild-type Ras signaling is sufficient to affect growth factor-induced cell migration *in vitro*, and will also address the contribution of MAPK and PI3K signaling to chemotaxis in this system.

5.2. FUTURE DIRECTIONS REGARDING WILD-TYPE RAS SIGNALING

5.2.i. Additional phenotypic consequences of silencing wild-type Ras expression

Depletion of wild-type Ras expression by siRNA results in the impaired two-dimensional growth of cancer cells with endogenous oncogenic *RAS* mutations (Figure 4.7, 4.8, 4.9). Future experiments should address whether wild-type Ras is necessary for growth factor-induced cellular migration *in vitro*, or for three-dimensional growth in agar or reconstituted basement membrane matrix. An inducible shRNA system would be helpful in addressing these questions, as we have found that constitutive expression of shRNAs targeting wild-type or oncogenic Ras are not well tolerated in cancer cell lines with oncogenic *RAS* mutations (data not shown).

It is also of great importance to test the requirement for wild-type Ras signaling *in vivo*. This could be achieved by modulating wild-type Ras expression in tumor xenografts or in various oncogenic Ras-driven transgenic mouse models of tumorigenesis.

5.2.ii. Specific inhibition of wild-type Ras activity

The results presented in Chapter 4 demonstrate that the depletion of wild-type Ras activity is sufficient to attenuate growth factor-induced MAPK and PI3K signaling and impede cell growth in cancer cell lines with oncogenic *RAS* mutations. It is unclear whether the specific inhibition of wild-type Ras activity (rather than the outright depletion of wild-type Ras expression) would produce the same effects. The results presented in Chapter 3 demonstrate that acute suppression of wild-type Ras activity by ephrin-A1-Fc stimulation is sufficient to attenuate growth factor-induced MAPK and PI3K signaling. However, as discussed above, phenotypic assays were not carried out under these experimental conditions. It could be argued that serum starvation serves to specifically inhibit wild-type Ras activity; as such, we have observed that wild-type Ras is predominantly GDP-bound in serum starved cells (Figure 4.2), and further, that serum starvation impedes the growth of cancer cells harboring oncogenic *RAS* mutations (Figure 4.1).

Another approach to address this question is to utilize an artificially membrane-targeted form of p120 RasGAP to constitutively promote the conversion of bound GTP to GDP on wild-type Ras (see Chapter 1.5.iii.). Experiments performed in the 1990s demonstrate that membrane-targeted p120 RasGAP antagonizes the transforming potential of ectopic oncogenic Ras in NIH3T3 cells (Clark et al., 1993; Huang et al., 1993). Importantly, this antagonism is dependent on the catalytic activity of p120 RasGAP. One interpretation of these results is that transformation depends on additive signals from both oncogenic and wild-type Ras, and that the constitutive down-regulation of wild-type Ras activity by membrane-targeted p120 RasGAP is sufficient to inhibit transformation by oncogenic Ras. Another interpretation is that wild-type Ras, in a GDP-bound form, directly antagonizes oncogenic Ras activity. Alternatively, a combination of these

mechanisms might be at play. We have tried to address these very questions using membrane-targeted p120 RasGAP, however we have found that ectopic expression of membrane-targeted p120 RasGAP (or the GAP-related domain of neurofibromin) is not well tolerated in cancer cell lines with oncogenic *RAS* mutations (data not shown). An inducible system of membrane-targeted p120 RasGAP expression is also not well tolerated, presumably due to the low levels of leaky expression. The fact that the expression of these ectopic proteins is not well tolerated in cancer cell lines harboring oncogenic *RAS* mutations suggests that they may in fact antagonize the growth or survival of these cells.

5.2.iii. Antagonism of oncogenic Ras signaling by wild-type Ras

In cancer cells harboring oncogenic *RAS* mutations, siRNA-mediated depletion of the wild-type Ras isoforms results in a slight increase in basal ERK phosphorylation (Figure 4.3), and suggests that wild-type Ras antagonizes oncogenic Ras signaling. The mechanism by which this antagonism occurs is not fully understood. In the basal state, wild-type Ras is predominantly GDP-bound. As discussed above, it is plausible that GDP-bound wild-type Ras inhibits oncogenic Ras signaling by sustaining unique and independent growth-inhibitory signaling pathways. To test this, future experiments could utilize the synthetic dominant negative Ras N17 mutant or membrane-targeted p120 RasGAP to maintain wild-type Ras in the GDP-bound state (See Chapter 1.5). Alternatively, it is possible that the different Ras isoforms might compete with each other for common regulators, effectors or proper localization. Additional experiments are required to fully address these issues.

5.3. FUTURE DIRECTIONS REGARDING ONCOGENIC RAS SIGNALING

5.3.i. Mechanism of EGFR rewiring

The exact molecular mechanism by which oncogenic Ras desensitizes EGFR signaling is not fully understood. The data presented here are consistent with reports indicating that high levels of MAPK signaling result in negative feedback to EGFR (Gan et al., 2010; Mirzoeva et al., 2009). It has been shown that activated ERK negatively regulates EGFR signaling by directly phosphorylating the receptor at T669, which in turn alters EGFR trafficking and turnover (Gan et al., 2010; Li et al., 2008). The data included in this study are consistent with those findings. However, several additional experiments could be performed to characterize alterations in EGFR signaling dynamics. For example, the binding and internalization of ligand to EGFR can be assessed using fluorescently labeled EGF in a live cell imaging system. It would be interesting to use this system to determine whether EGF binding and internalization is altered upon ectopic oncogenic Ras expression, or conversely, upon depletion of oncogenic Ras with siRNA. To demonstrate a correlation between a reduction in EGFR signaling and high MAPK pathway activity, additional controls should include conditions in which MAPK signaling is suppressed or enhanced by treatment with a MEK inhibitor or by ectopic expression of constitutively active MEK, respectively.

Recent studies indicate that EGFR is mislocalized in cells expressing oncogenic Ras (van Houdt et al., 2010). To confirm and extend upon these findings, immunofluorescence analysis of EGFR localization could be performed in cells expressing ectopic oncogenic Ras and in cancer cells in which oncogenic Ras is depleted by siRNA. It should also be determined whether EGFR localization is altered under conditions in which MAPK signaling is suppressed or enhanced by treatment with a MEK inhibitor or by ectopic expression of constitutively active MEK, respectively. An alternate approach to examine EGFR localization could involve using fluorescence-activated cell sorting to examine cell surface levels of EGFR. These experiments will help indicate

whether alterations in EGFR localization or signaling dynamics contribute to the desensitization of EGFR signaling observed in cancer cells harboring oncogenic *RAS* mutations.

5.3.ii. Modulation of signaling from other receptor tyrosine kinases

The data presented here are consistent with a model in which oncogenic Ras-driven MAPK pathway activity attenuates EGFR signaling. It is not known whether oncogenic Ras specifically desensitizes EGFR signaling, or if additional upstream receptor tyrosine kinases are affected as well. Preliminary data show that high levels of ectopic oncogenic K-Ras and N-Ras expression attenuates GM-CSF signaling in macrophages (J. Xu and K. Shannon, unpublished). Therefore, oncogenic Ras might very well engage a program that results in the global deactivation of upstream receptor tyrosine kinase signaling.

An anti-phosphotyrosine receptor antibody array can be utilized to determine whether receptor tyrosine kinases other than EGFR are desensitized by oncogenic Ras (Chandarlapaty et al., 2011). Serum stimulation experiments could be performed on cells in which MAPK activity is induced by ectopic oncogenic Ras expression or attenuated by either siRNA-mediated depletion of oncogenic Ras or by treatment with a MEK inhibitor. Lysates prepared from these conditions could then be applied to anti-phosphotyrosine receptor arrays, which assess the tyrosine phosphorylation content of 42 different receptor tyrosine kinases. The results of these experiments will establish whether the activation of various receptor tyrosine kinases is feedback inhibited by oncogenic Ras.

5.3.iii. Rewiring of growth factor signaling by high MAPK pathway activity and implications for genetic developmental disorders

Activating mutations in various components of the Ras/MAPK signaling cascade have recently been identified in patients with related genetic developmental disorders (Schubbert et al., 2007).

Germline gain-of-function mutations in *KRAS*, *BRAF*, *MEK1*, and *MEK2* have been observed in patients with Cardiofaciocutaneous (CFC) syndrome (Niihori et al., 2006; Rodriguez-Viciana et al., 2006). Additionally, activating mutations in MAPK pathway components are present in patients with similar neuro-cardio-facial-cutaneous syndromes, including Noonan, LEOPARD, and Costello syndromes (Aoki et al., 2008; Denayer et al., 2008). These syndromes share numerous phenotypes including short stature, developmental delay, characteristic facial features, skin abnormalities, cardiac defects, and a predisposition to malignancies (Denayer et al., 2008).

The data presented here show that oncogenic Ras attenuates growth factor-induced signaling, and does so by the induction of high MAPK pathway activity. Future experiments should characterize the Ras/MAPK pathway mutations identified in these genetic developmental disorders to determine whether they attenuate signaling from upstream receptor tyrosine kinases as well. It is tantalizing to speculate that the feedback inhibition of growth factor signaling by high MAPK pathway activity might have a causal role in the growth defects observed in these disorders.

5.4. CONCLUDING REMARKS

In conclusion, the work presented here uncovers several interesting aspects of wild-type and oncogenic Ras biology that have been previously underappreciated. This work demonstrates a critical role for wild-type Ras in mediating growth factor-induced signaling and sustaining the proliferation of cancer cells harboring oncogenic *RAS* mutations. The discovery that wild-type Ras contributes to the overall signaling output in cells harboring oncogenic *RAS* mutations is a novel and surprising finding, and may uncover less obvious modes of targeting the pathway that have yet to be exploited. Additionally, the finding that oncogenic Ras rewires EGFR signaling in a MEK-dependent fashion might help explain the observation that *KRAS* mutant tumors are often resistant to EGFR-based therapies, and could lead to new strategies for treating cancers that contain oncogenic *RAS* mutations.

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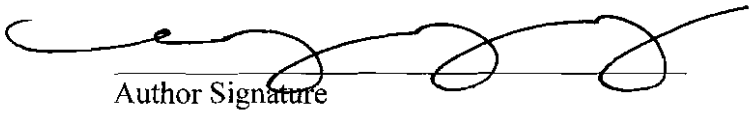

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